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Oncomodulin protein interactions: Structural and functional consequences.

Elizabeth Johanna. Palmer
University of Windsor

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ONCOMODULIN PROTEIN INTERACTIONS:

STRUCTURAL AND FUNCTIONAL CONSEQUENCES

by

© Elizabeth Johanna Palmer

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ABSTRACT

ONCOMODULIN PROTEIN INTERACTIONS:

STRUCTURAL AND FUNCTIONAL CONSEQUENCES

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Oncomodulin is an oncodevelopmental Ca^{2+} binding protein of the Troponin C superfamily. The reactivity of the cys-18 thiol of oncomodulin has been probed with 5,5'-dithiobis(2-nitrobenzbate) (DTNB). The kinetics of the reaction indicate that the thiol is ~10 times more accessible in the presence of Ca^{2+} than in EGTA. In addition, oncomodulin has the ability to dimerize via intermolecular disulfide bond formation in vitro. The kinetics of dimerization indicate that the second order rate constant for the formation of dimer is ~6 fold higher than that observed for the reaction of the free thiol with DTNB, perhaps an indication that intermolecular electrostatic attractions precede and facilitate dimerization. The disulfide linked dimer of oncomodulin appears to be more calmodulin like in structure than the monomer since the dimer displayed affinity for the amphiphilic peptide melittin in the same range as calmodulin. To this end, oncomodulin dimer was shown to activate two calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, with activity constants of 63nM

and 1nM, respectively, indicating that these enzymes have different domain requirements for activation. In addition, oncomodulin was shown to interact with glutathione reductase (GSSGRase) (from bovine intestinal mucosa and rat liver) in a calcium dependent manner. This is evidenced by the fact that glutathione reductase binds to oncomodulin Sepharose only in the presence of calcium and was eluted by the application of EGTA. The inclusion of increasing amounts of reduced oncomodulin in the GSSGRase assay resulted in inhibition of the enzyme with 50% inhibition occurring at ~1E-5M. The closely related carp and rabbit parvalbumins as well as calmodulin had no effect on GSSGRase activity. This is the first report of a regulatory role for oncomodulin which is not shared by calmodulin. The kinetics obtained illustrate that reduced oncomodulin behaves as a noncompetitive inhibitor of GSSG utilization by GSSGRase. In addition, oxidized oncomodulin can act as a ~~substrate for~~ GSSGRase in vitro, as evidenced by SDS-PAGE analysis of the reaction products. These results suggest that oncomodulin monomer is involved in the maintenance of glutathione levels in tumour cells. Therefore, it appears from these studies that oncomodulin monomer and dimer are both important in the metabolism of neoplastic cells, each possessing different regulatory roles, some of which seem to be oncomodulin specific effects. Studies performed on the stability of oncomodulin dimer to 10mM GSH indicate that the dimer may not

survive the reducing conditions present intracellularly. Hence, the functions of oncomodulin dimer may lie in the extracellular environment.

dedicated to my husband

and my best friend,

Richard

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ABBREVIATIONS

BIM	bovine intestinal mucosa
BME	2-mercaptoethanol
BSA	bovine serum albumin
CaM	calmodulin
cAMP	3',5' cyclic adenosine monophosphate
CaN	calcineurin
cPV	carp parvalbumin
dansyl	5-(dimethylamino)-1-napthalenesulfonyl
DEAE	diethylaminoethyl
DNS-CaM	dansylcalmodulin

DTNB	5,5'-dithiobis(2-nitrobenzoate)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EGTA	ethylene glycol bis (beta-aminoethyl ether) N,N,N',N'-tetraacetate
FAD	flavin adenine dinucleotide
GSH	reduced glutathione
GSSG	oxidized glutathione
GSSGRase	glutathione reductase
HPSEC	high performance size exclusion chromatography
M_r	relative molecular weight
M_w	molecular weight

NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
ONC	oncomodulin
PAGE	polyacrylamide gel electrophoresis
PDE	phosphodiesterase
pNPP	para-nitrophenyl phosphate
RL	rat liver
SAMONC	S-iodoacetamide labelled oncomodulin
SDS	sodium dodecyl sulfate (lauryl sulfate)
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TnC	troponin C

Tris-HCl	tris (hydroxymethyl) aminomethane

INTRODUCTION

1. Historical Perspective of Oncomodulin

A low molecular weight Ca^{2+} binding protein from neoplastic tissues was first reported by J. P. MacManus in 1979 (MacManus, 1979). The protein was detected in the cytosol of rat Morris hepatomas 5123tc, 7288 and 7795, but could not be detected in adult or fetal liver tissue. The amount of protein detected was greatest in Morris hepatoma 5123tc and least in Morris hepatoma 7795 but did not appear to be directly related to the rate of growth of the tumour. The protein had an apparent molecular weight of less than 12500D and was distinct from calmodulin, the ubiquitous Ca^{2+} binding protein of normal tissues (Cheung, 1980; Wang et al., 1985). In 1980, this protein was isolated from rat Morris hepatoma 5123tc and found to have a molecular weight of ~11000D and an isoelectric point of 3.9 (MacManus, 1980). It was found to bind 2 moles of Ca^{2+} per mole of protein and appeared to be quite similar in amino acid composition to the parvalbumins. This protein was later named oncomodulin by MacManus and coworkers in 1982 (Boynton et al., 1982).

The development of an antiserum against oncomodulin enabled the detection of oncomodulin in other tumour sources (MacManus, 1981a). The immunoassay allowed the detection of

oncomodulin in chemically induced early neoplastic rat liver nodules, rat Morris hepatomas and mouse sarcomas (MacManus, 1981). To date, oncomodulin has been detected in several tumours from various sources including virally transformed rat kidney cells (Durkin et al, 1983), tumours from guinea pigs (MacManus and Whitfield, 1983) and also human tumours of bladder, brain, breast, cervix, colon, kidney, liver, lung, skin and uterus (MacManus et al., 1984). In addition, this protein was detected in the extraembryonic tissues of the fetal rat: in the inner placenta (labyrinth), the outer placenta (basal zone and decidua), parietal and visceral yolk sac and the amnion (Brewer and MacManus, 1985). The amount of oncomodulin present in the placenta was found to increase throughout the gestational period. No oncomodulin was detected in the embryo. This finding indicates that oncomodulin is an oncodevelopmental protein, being expressed during development and upon neoplastic transformation.

2. The Troponin C Superfamily

The Troponin C superfamily of proteins is characterized by low molecular weights and Ca^{2+} binding properties (Kretsinger, 1980). Each member of the family binds Ca^{2+} in calcium binding domains which are termed EF hands (FIGURE 1). These structures consist of two alpha helices at right angles

FIGURE 1

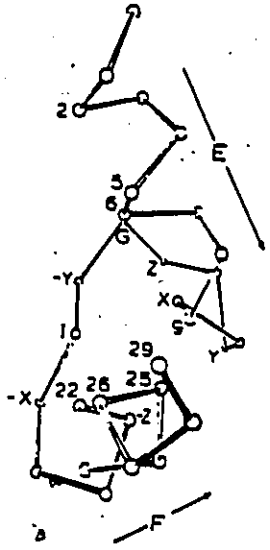


FIGURE 1: Secondary Structure of EF Hand. The E and F alpha helices are indicated as are the six ligands (designated X, Y, Z, -X, -Y, -Z) which bind the Ca^{2+} ion. The highly conserved glycine (G) and isoleucine (I) are also illustrated. (Reproduced from Kretsinger, 1980).

to each other which are linked by a Ca^{2+} binding loop of ~12 residues. There is a great deal of homology between the various members especially in the calcium binding regions of the proteins. The proteins tend to be low in or devoid of tryptophan residues and have a high phenylalanine to tyrosine ratio (Klee and Vanaman, 1982).

The members of the Troponin C superfamily include Troponin C, calmodulin, brain S100 protein, vitamin D dependent protein and the parvalbumins. Troponin C is an integral part of the Troponin complex of muscle tissue which is responsible for the Ca^{2+} dependent contraction of muscle fibers (Herzberg et al., 1986). S100 proteins (A and B) are particularly abundant in brain tissue and are thought to be multifunctional effector proteins, involved in the development and function of neural cells (Baudier and Cole, 1988; Zimmer and Van Eldik, 1986). Vitamin D dependent protein is found throughout the intestine and is involved in the translocation of Ca^{2+} across the intestinal wall (Szebenyi and Moffat, 1987). The role of parvalbumins is unclear at this time but they appear to be a storage depot for calcium in muscle tissue (Heizmann and Celio, 1987). Calmodulin is a ubiquitous protein which is present in virtually all cells. Like Troponin C, calmodulin undergoes a conformational change upon exposure to calcium (Ikura, 1983; Ikura et al., 1984). This change in conformation results in the binding of calmodulin to several target proteins. As a

result the activity of target enzymes is controlled by the action of calmodulin. These enzymes include cyclic nucleotide phosphodiesterase from various sources (Rossi et al., 1988, Hanson et al., 1988, Sharma and Wang, 1988, Kincaid and Vaughan, 1988, Purvis and Rui, 1988), calcineurin phosphatase (Hurwitz et al., 1988), phosphofructokinase (Mayr, 1987), myosin light chain kinase (Adelstein and Klee, 1981) adenylate cyclase (Veigl et al., 1984), glycogen synthase kinase (Payne and Soderling, 1980) and the insulin receptor tyrosine kinase which also phosphorylates calmodulin (Laurino et al., 1988). The structure of calmodulin has been determined and illustrates an overall dumbbell shape which is shared by Troponin C (FIGURE 2) (Babu et al., 1985 and 1988; Herzberg and James, 1985; Heidorn and Trewhella, 1988). Each protein possesses four calcium binding domains, two of which are located in each end of the dumbbell joined by a long helical segment. It is this intervening helical region of calmodulin which is thought to be the site of interaction of calmodulin with its target proteins (Putkey et al., 1988).

3. The Structure of Oncomodulin

Oncomodulin is a low molecular weight calcium binding protein which is heat stable and has an isoelectric point of

FIGURE 2

FIGURE 2: Tertiary Structure of Calmodulin and Troponin C. The crystal structures of calmodulin and Troponin C are illustrated. Both proteins exhibit an overall dumbbell shape. Calcium ions are indicated by filled circles. (Reproduced from Heidorn and Trewhella, 1988).

3.9 (MacManus, 1980). The amino acid composition of oncomodulin illustrates that the protein is devoid of tryptophan residues and has a high phenylalanine to tyrosine ratio. These characteristics are all common to the members of the Troponin C superfamily (Kretsinger, 1980). The primary structure of oncomodulin was determined in 1983 (FIGURE 3) (MacManus et al., 1983). The sequence illustrates remarkable homology to the beta parvalbumin subclass of the Troponin C superfamily. A comparison of their primary structures is given in FIGURE 4. Carp beta parvalbumin (pI = 4.25) has 52 amino acids in identical positions to oncomodulin including the solitary thiol at position 18 (Kretsinger, 1980). Rabbit skeletal muscle beta parvalbumin is also quite homologous, having 50 amino acids in identical positions to rat oncomodulin (Capony et al., 1976). Each of these proteins contains two calcium binding domains which are characteristic of the Troponin C superfamily. The second Ca^{2+} binding loop is virtually identical in all three proteins. Oncomodulin is therefore considered to be a parvalbumin like protein in structure.

The complete complementary DNA for oncomodulin has recently been determined (Gillen et al., 1987). Although the sequence showed little homology to the nucleotide sequences of several other members of the Troponin C superfamily, the sequence illustrated 59% homology to that for parvalbumin (Epstein et al., 1986). This further illustrates the close

FIGURE 3

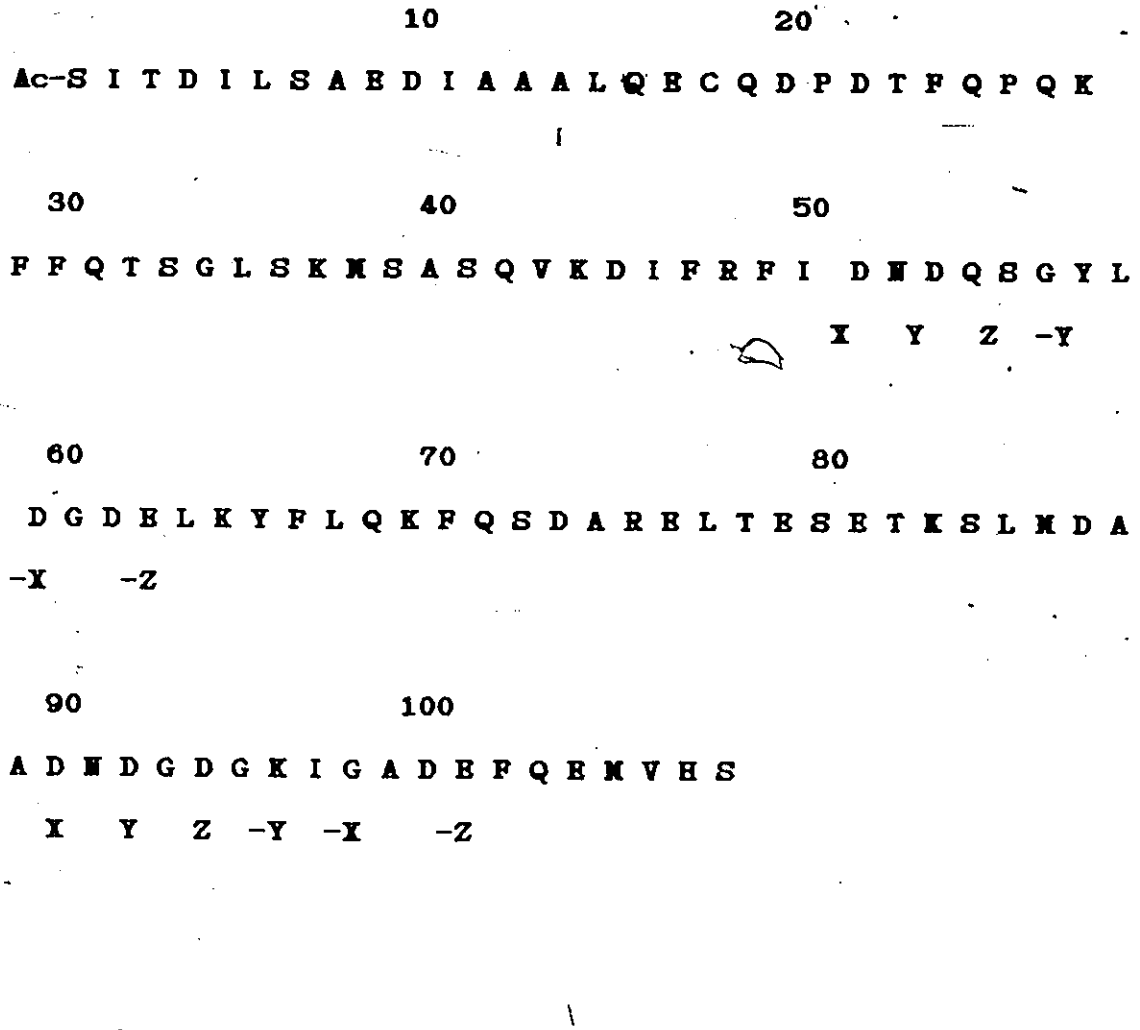


FIGURE 3: Primary Structure of Oncomodulin. The one letter code is utilized to represent the amino acid sequence. The coordinates X, Y, Z, -X, -Y, -Z refer to the calcium binding ligands established by Kretsinger (1980). (MacManus et al., 1983)

FIGURE 4

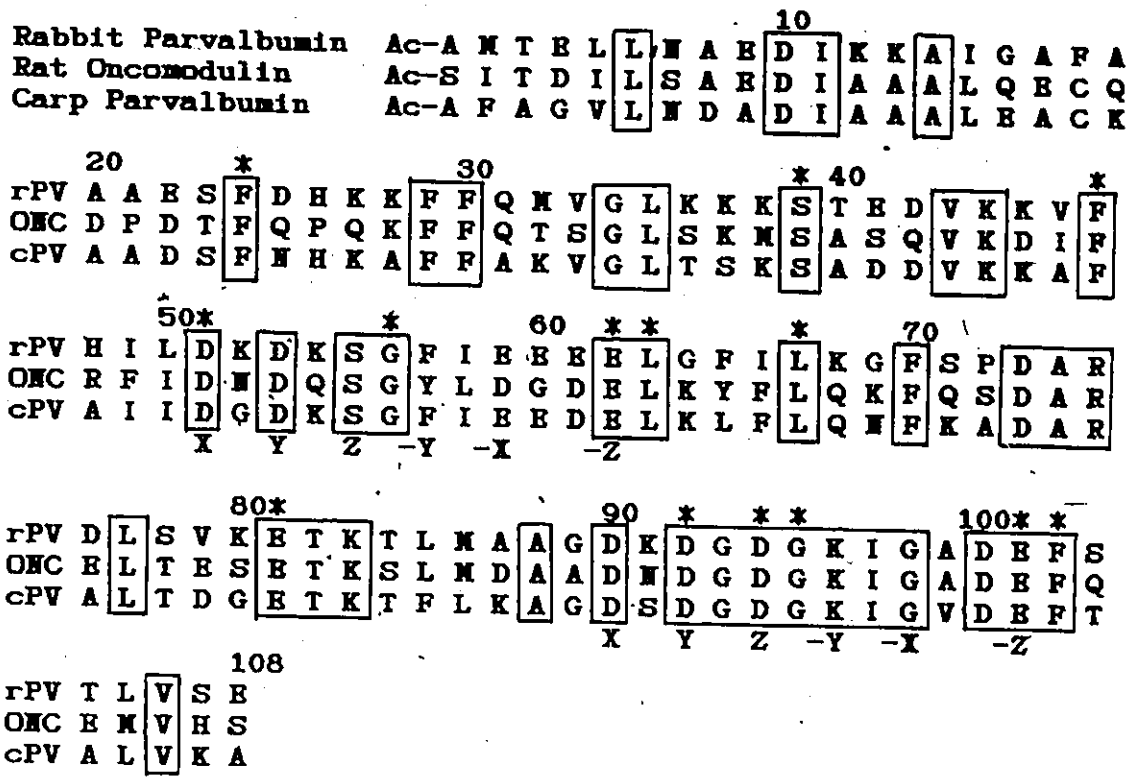


FIGURE 4: Comparison of Primary Structure of Rat Oncomodulin, Carp Beta Parvalbumin (pI 4.25) and Rabbit Parvalbumin. The one letter code is utilized to represent the amino acid sequence. The coordinates X, Y, Z, -X, -Y, -Z refer to the calcium binding ligands established by Kretsinger (1980). Rabbit parvalbumin sequence is taken from Capony et al., 1976; oncomodulin sequence from MacManus et al., 1983; carp parvalbumin from Coffee and Bradshaw, 1973. Asterisks indicate amino acids which are common to all members of the Troponin C superfamily. Boxes indicate identical residues between the parvalbumin and oncomodulin.

relationship between oncomodulin and its parvalbumin counterparts in comparison to other members of the Troponin C superfamily.

The gene for oncomodulin from rat Morris hepatoma has also recently been cloned into E. coli by MacManus and coworkers (MacManus et al., 1989). This accomplishment has enabled the large scale preparation of oncomodulin, ~50 fold more oncomodulin than that obtained from Morris hepatoma 5123tc which is the richest source to date. This greatly facilitates research into possible areas of oncomodulin regulation. In addition, the possibility of selective mutation is now a reality and can be utilized to study the interactive properties of oncomodulin with its target proteins.

4. Functional Differences Between Oncomodulin and Carp Beta Parvalbumin

The primary structure of oncomodulin shows a great deal of homology to the beta parvalbumin subclass of the Troponin C superfamily (MacManus et al., 1983; Heizmann, 1987). All members of the Troponin C superfamily are distinguished by possessing Ca^{2+} binding loops located between two alpha helices which are termed EF hands (Klee and Vanaman, 1982). Conformational studies on oncomodulin have suggested that

oncomodulin undergoes a conformational change in the presence of Ca^{2+} but not Mg^{2+} (MacManus et al., 1984). These conformational changes were detected using intrinsic probes (tyrosine and phenylalanine) which are located in the CD and EF metal binding domains of oncomodulin. Their results indicate that the conformational changes in the CD loop were Ca^{2+} specific whereas those observed in the EF loop were induced by either Ca^{2+} or Mg^{2+}. The pairing of one calcium site with one calcium/magnesium site is unusual and has not been observed in the parvalbumins (MacManus et al., 1984; Williams et al., 1987; Henzl et al., 1986).

An additional study performed in our laboratory exploited the solitary thiol at position 18 of oncomodulin's primary structure which was labelled with the thiol-specific reagent N-dansylaziridine (N-DAZ) (Mutus et al., 1985a). N-dansylaziridine is a fluorescent reagent which exhibits high selectivity for protein thiols, forming highly fluorescent N-dansyl-S-2-aminoethyl protein adducts (Mutus et al., 1981; Scouten et al., 1974). The study involved the comparison of labelled oncomodulin to labelled carp beta parvalbumin (pI = 3.95) which also possesses a single thiol at position 18 of its primary structure. In the presence of 0.1mM Ca^{2+}, the incubation of oncomodulin with N-DAZ resulted in approximately 1.0 mole of reagent incorporated per mole of protein; this ratio decreased approximately 35% when the modification was performed in the absence of Ca^{2+}. This

cation dependence of thiol reactivity was not mimicked by the carp parvalbumin. The emission spectrum of N-DAZ labelled oncomodulin in the presence of 0.05mM Ca^{2+} and physiological magnesium ion (2mM) was decreased by approximately 20%, with no apparent shift in the emission wavelength maximum. The quenching which was observed was complete at a Ca^{2+} oncomodulin molar ratio of 1:1. Therefore the conformational change observed in our study, where the label is incorporated into the AB loop of the protein was a result of the binding of Ca^{2+} to the CD loop. Under identical conditions, no changes were observed in the fluorescence emission spectrum of the labelled parvalbumin. This was the first report of a long range structural perturbation of oncomodulin induced by the binding of Ca^{2+} and was an essential find if one is to consider oncomodulin as a calcium binding regulatory protein.

Additional studies have been performed utilizing lanthanide ions to study the affinity of the metal binding sites of oncomodulin versus parvalbumin (Williams et al., 1987; Henzl et al., 1986; Henzl and Birnbaum, 1987 and 1988). The characteristics of lanthanide ion bound to the CD metal binding site of oncomodulin were significantly different than those observed at the EF site and on parvalbumin. The CD site of oncomodulin was found to possess little preference for europium ion compared to calcium whereas the EF site as well as both metal binding domains of parvalbumin displayed a large preference for Eu^{3+} (Henzl and Birnbaum, 1987 and

1988). This change in degree of preference for metal ions was also observed with Tb^{3+} (Henzl et al., 1986) Lu^{3+} and Yb^{3+} (Williams et al., 1987). This further illustrated that the CD metal binding site of oncomodulin is structurally different from the EF site and the two sites present on parvalbumin. Calcium binding site III of Troponin C also illustrates a lower preference for calcium relative to the parvalbumins as does the CD site of oncomodulin (Williams et al., 1987). The EF metal binding domain of oncomodulin and parvalbumin have 11 of 12 amino acids in common whereas the homology at the CD metal binding domain drops to 7 of 12 amino acids (MacManus et al., 1983). One of the altered residues is that at the -X Ca^{2+} binding ligand which in oncomodulin and Troponin C is aspartic acid compared to glutamic acid in the parvalbumin (Williams et al., 1987). These amino acid substitutions result in an alteration in the selectivity of the CD binding site of oncomodulin for calcium. Recent work by MacManus and coworkers (1989) illustrates that if the aspartic acid residue of the CD site of oncomodulin is replaced by glutamic acid via gene mutation, the calcium specificity is abolished; the mutant oncomodulin was found to exhibit a conformational change in the presence of magnesium (MacManus et al., 1989).

Therefore, oncomodulin, although similar to the parvalbumins in primary structure, displays a Ca^{2+} specific conformational change much like calmodulin and Troponin C

which the parvalbumin can not mimic and as a result possesses the flexibility necessary for it to act as a Ca^{2+} binding regulatory protein in neoplastic tissues.

5. Function of Oncomodulin Compared to Calmodulin

To date oncomodulin has been shown to mimic the action of calmodulin in a small number of systems. In 1981, oncomodulin was demonstrated to activate rat heart cyclic nucleotide phosphodiesterase in a Ca^{2+} dependent manner (MacManus, 1981). Half maximal activation occurred at approximately 5E-8M oncomodulin compared to ~1E-9M for calmodulin. Rat parvalbumin was unable to activate the enzyme under identical conditions. Calmodulin dependent bovine heart phosphodiesterase was also activated by oncomodulin with half maximal activation occurring at ~2E-7M (Mutus et al., 1985b). Calmodulin dependent bovine brain phosphodiesterase activity was not affected by the addition of oncomodulin (Mutus et al., 1985b; Klee and Heppel, 1984).

Normal cells are known to require millimolar levels of extracellular calcium for proliferation (Boynton et al., 1980). The addition of calmodulin to rat liver cell cultures, however, results in the stimulation of DNA synthesis (Boynton et al., 1980). Oncomodulin was capable of mimicking this effect by stimulating DNA synthesis in Ca^{2+}

deprived non-neoplastic rat liver cells (Boynton et al., 1982). Again, this effect was not observed in the presence of the highly homologous rat parvalbumin.

These findings suggest that although oncomodulin is closely related to the beta parvalbumins in primary structure, oncomodulin is capable of substituting for calmodulin in some calmodulin dependent processes. Therefore, the secondary structure of oncomodulin must resemble that of calmodulin more than parvalbumin in order for oncomodulin to interact with calmodulin target proteins, perhaps providing a clue to the physiological role of oncomodulin. However, the concentrations required for half maximal activation of the calmodulin dependent enzymes studied appear to be too high to be of physiological relevance when compared to the levels of calmodulin required. Further study is thus necessary to determine the role of oncomodulin in vivo.

6. Melittin as a Model of the Calmodulin Target Protein Binding Domain

Melittin is a cytotoxic peptide isolated from bee venom (Terwilliger and Eisenberg, 1982; Habermann, 1972). The peptide is 26 amino acids in length and contains two basic alpha helices joined by a bend to give the overall shape of a

bent rod or a hairpin (Terwilliger and Eisenberg, 1982; Bazzo et al., 1988). The amino acids are arranged in the helix in such a way that the hydrophobic amino acids are on one face while the hydrophilic amino acids are on the other; this arrangement is termed amphiphilic. Melittin displays Ca^{2+} dependent high affinity for several members of the Troponin C superfamily including calmodulin (K_d = 1-3nM: Cox et al., 1985; Maulet and Cox, 1983; Comte et al., 1983), Troponin C (Steiner and Norris, 1987) and S100b protein (Baudier et al., 1987). Mastoparan X, another basic amphiphilic cytotoxic peptide is also capable of binding to calmodulin with Ca^{2+} dependent high affinity (Sanyal et al., 1988; Cachia et al., 1986; Van Eyk and Hodges, 1987).

Several reports indicate the affinity of melittin for calmodulin. Melittin conjugated to Sepharose has been used for the purification of calmodulin (Kincaid, 1987). Free melittin has been shown to inhibit the stimulation of bovine brain cyclic nucleotide phosphodiesterase by calmodulin (Cox et al., 1985). In addition, melittin antibodies are able to recognize the calmodulin binding domain on calmodulin target proteins resulting in the inhibition of calmodulin target protein binding (Kaetzel and Dedman, 1987). Recent studies have illustrated that melittin is capable of binding to fragments of calmodulin and indicate that the peptide binding domain is located in the C terminal half of calmodulin (residues 78 - 148) (Sanyal et al., 1988). ^1H NMR studies of

calmodulin with melittin illustrate that the binding of melittin causes structural alterations in both the C and N terminal halves of calmodulin (Seeholzer et al., 1986). As a conclusion to these findings, melittin is thought to possess the minimum structural requirements necessary for the calmodulin target protein interaction (Cox et al., 1985). Melittin may therefore be utilized as a model of the calmodulin target protein binding domain and can be used to detect a structurally similar domain in other proteins.

Several reports have been published illustrating that calmodulin has several modes of interaction with its target proteins. For example the 78 - 148 fragment of calmodulin (where calmodulin has been cleaved in the central helix connecting the calcium binding domains) although able to bind to phosphodiesterase is unable to activate the enzyme (Newton et al., 1984; Minowa et al., 1988). However, the same fragment is able to activate phosphorylase kinase but with somewhat lower affinity than intact calmodulin (Kuznicki et al., 1981). This 78 - 148 fragment failed to stimulate calcineurin (Newton et al., 1984). A subsequent report by Ni and Klee (1985) utilized calmodulin fragments coupled to Sepharose. Fragment 78 - 148 was found to bind calcineurin, cAMP phosphodiesterase and cAMP dependent protein kinase from bovine brain in a Ca^{2+} dependent manner. Only the phosphodiesterase and cAMP dependent protein kinase were observed to interact with the 1 - 77 fragment (Ni and Klee,

1985). Studies with a synthetic gene product in which the central helix of calmodulin had been altered indicated that this helix was involved in the activation of myosin light chain kinase and NAD kinase as activation of myosin light chain kinase with the synthetic calmodulin was decreased and the activation of NAD kinase was abolished (Craig et al., 1987). Modification of various lysine residues of calmodulin also have differential effects on target enzymes (Thiry et al., 1980; Giedroc et al., 1985; Guerini et al., 1987; Manalan and Klee, 1987; Mann and Vanaman, 1988). A recent report on the in vitro nonenzymatic glycation of calmodulin lysines also supports the conclusion of different sites of interaction on calmodulin for target proteins (Kowluru et al., 1989).

Studies on the interaction of melittin with calmodulin indicate that complex formation involves the central helix of calmodulin connecting the two dumbbell lobes (Caday and Steiner, 1986). The target proteins of calmodulin exhibit extensive variation with respect to the site(s) of interaction on calmodulin. Therefore the use of melittin to study the interaction of calmodulin with target proteins must take into account the diversity of interactions between calmodulin and its target proteins and as a result, melittin will be a model for only some of the target enzyme binding domains. In addition, melittin is capable of interacting with calmodulin in the absence of calcium, a property not

shared by the majority of calmodulin target proteins (Cox, 1988; Milos et al., 1987).

7. Alterations in Metabolism of Neoplastic Cells Compared to Normal Cells

There are several characteristics which distinguish neoplastic cells from their normal counterparts. The most obvious of these alterations is the uncontrolled growth which occurs upon neoplastic transformation. The mechanisms by which these transformed cells maintain this rate of growth is unclear at this time. The cells are characterized in part by their ability to grow in a semi-solid medium illustrating an anchorage independence not observed in normal cells and also their loss of contact inhibition which normally arrests the growth of normal cells in vitro (Veigl et al., 1984). In addition, the profile of glycoproteins observed is modified in neoplastic systems. Recently there has been much study in this area concerning a plasma membrane P-glycoprotein of M$_r$ 170000 - 180000 (Sugimoto and Tsuruo, 1987; Jongsma et al., 1987; Fairchild et al., 1987). This glycoprotein is thought to afford multidrug resistivity to the neoplastic cell as most if not all multidrug resistant neoplasms overexpress this particular glycoprotein (Jongsma et al., 1987). This glycoprotein appears to be involved in the energy dependent

efflux of various compounds and as a result, in the neoplastic state, transports drugs out of the neoplasm (Cordon-Cardo et al., 1989). This efflux can be blocked by the addition of several steroids which compete with the antineoplastic drugs for the P-glycoprotein suggesting a means of overcoming the observed multidrug resistance (Yang et al., 1989).

Several other metabolic alterations in neoplasms are well documented and are discussed in the subsequent subsections.

A. Calcium Metabolism

The calcium regulation of neoplastic tissues is different from that of normal cells. Normal cells require millimolar levels of extracellular free calcium for proliferation. A medium depleted of extracellular Ca^{2+} inhibits DNA synthesis and thus results in the cells being locked in the G_1 phase of their growth-division cycle (FIGURE 5) (Swierenga et al., 1980). Calmodulin is also involved in the initiation of DNA synthesis: the addition of purified calmodulin to Ca^{2+} deprived rat liver cell cultures stimulated DNA synthesis (Boynton et al., 1980). In addition, the level of calmodulin in rat liver cells has been shown to rise transiently before the initiation of DNA synthesis both in vivo and in vitro (Boynton et al., 1982). The addition of calmodulin inhibitors to the medium, such as

FIGURE 5

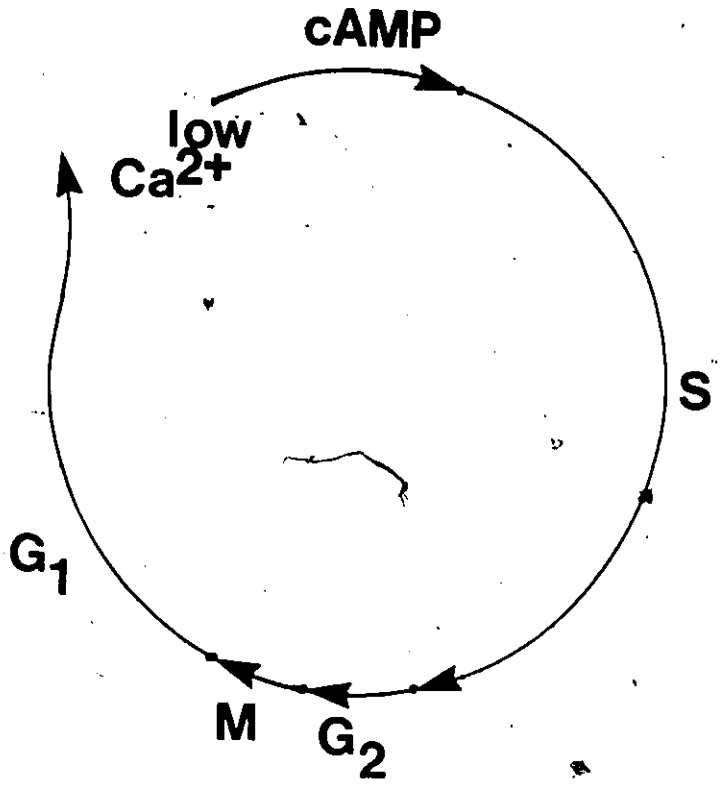


FIGURE 5: Inhibition of the Growth Division Cycle by Depleted Extracellular Ca $^{2+}$. Illustrated is a schematic representation of the blockage of DNA synthesis in liver cells by low extracellular free calcium ion concentration. The normal pre-replicative cAMP surge is illustrated. S: DNA synthesis stage; G_2: growth phase; G_1: growth phase; M: mitosis phase. (Swierenga et al., 1980)

trifluoperazine, prevents the initiation of DNA synthesis, an effect which can be reversed by the addition of excess calmodulin (Boynton et al., 1980).

Tumour cells have lost the dependence on extracellular free calcium for proliferation (Boynton and Whitfield, 1976; Boynton et al., 1977; Swierenga et al., 1978). Neoplastic cells from various sources have been shown to proliferate indefinitely in low calcium media which do not support the growth of normal cells (Boynton and Whitfield, 1976; Boynton et al., 1977). This loss of extracellular calcium dependence for proliferation was suggested by Swierenga and coworkers to be an indicator of tumorigenicity in vitro (Swierenga et al., 1978). The intracellular levels of calcium are also higher in neoplastic tissues than in normal cells (~1.5 to 2 fold) (Tsuruo et al., 1984). An additional alteration in neoplastic cells compared to their normal counterparts is in the level of intracellular calmodulin. Several types of human, rodent and avian tumours have been illustrated to possess two to four times more calmodulin than the normal tissue (VanEldik and Watterson, 1979; Watterson et al., 1976; Wei and Hickie, 1981; Takemoto et al., 1983; Nakajo et al., 1983; Brinkley et al., 1981; Viegl et al., 1984; MacManus et al., 1981).

Additive to all these changes in calcium regulation in neoplastic tissues is the expression of a second Ca^{2+} binding modulatory protein, namely oncomodulin. Like calmodulin,

oncomodulin has been shown to stimulate DNA synthesis in calcium deprived non-neoplastic rat liver cells in culture (Boynton et al., 1982). The initiation of DNA synthesis by oncomodulin was also inhibited by the presence of trifluoperazine, the calmodulin inhibitor, but as with the case of calmodulin, excess oncomodulin could override the effect of trifluoperazine. The effect of oncomodulin was seen at concentrations ranging from 1E-9M to 5E-6M, being most effective in the 1E-9 to 1E-7M range. The range of calmodulin necessary to affect stimulation of DNA synthesis was actually higher, with concentrations greater than 1E-8M calmodulin necessary.

As a result of these studies it has been suggested that the disappearance of extracellular calcium dependence for proliferation upon neoplastic transformation is a result of a combination of factors. The levels of calmodulin and intracellular calcium are both increased ~2 fold. Additive to this is the expression of oncomodulin, which appears to be superior to calmodulin at stimulating DNA synthesis in the calcium deprived state. These factors acting together may result in permanent activation of DNA synthesis leading to the unrestrained growth observed in the neoplastic state (Boynton et al., 1982).

B. Cyclic Nucleotide Metabolism

Cyclic nucleotide metabolism is altered in neoplastic

tissues compared to their normal counterparts (Watterson et al., 1976; Swierenga et al., 1980; Boynton and Whitfield, 1983). During the initial stages of neoplastic transformation, there is an increase in adenylate cyclase activity (Boynton and Whitfield, 1983). However, there are conflicting reports; neoplastic cells have been illustrated to have increased or decreased levels of adenylate cyclase, cyclic nucleotide phosphodiesterase, and type I and II cAMP dependent protein kinase activities (Boynton and Whitfield, 1983).

Cyclic AMP levels have been demonstrated to be lower in virally transformed chicken embryo fibroblasts than their normal counterparts (Otten et al., 1972). By artificially increasing intracellular cAMP levels, the transformed cells exhibited properties characteristic of the normal cell. This decrease in cAMP levels in neoplastic tissue may in part be due to the increased levels of calmodulin present in the cell which would further activate cyclic nucleotide phosphodiesterase and thus decrease cAMP levels (Swierenga et al., 1980; Watterson et al., 1976; MacManus et al., 1981).

The initiation of DNA synthesis in normal cells is preceeded by an early and late cAMP surge and an increase in the levels of cAMP dependent and independent protein kinase levels (Swierenga et al., 1980; Boynton and Whitfield, 1983). In addition, calmodulin levels also increase during the pre-DNA synthesis stage both <u>in vivo</u> and <u>in vitro</u> (Soriano et

al., 1988; MacManus et al., 1981; Pinol et al., 1988). The later cAMP surge is inhibited by the depletion of extracellular calcium and DNA synthesis is blocked (Boynton et al., 1982). Low concentrations of exogenous cAMP or its analogue, N^6,2'-O-dibutyryl cAMP, are able to overcome this block and DNA synthesis in calcium deprived cells is initiated to some degree (Boynton and Whitfield, 1979). Under milder cases of calcium deprivation the cAMP surge is not inhibited but DNA synthesis still does not occur (Swierenga et al., 1980). This suggests that both calcium and cAMP are integral in the initiation of DNA synthesis. The alterations in cAMP levels in neoplastic tissues coupled with the alterations in calcium metabolism discussed previously suggest that the synthesis of DNA is independent of the calcium - cAMP signal observed to precede DNA synthesis in normal cells. Possible mechanisms for this are numerous including altered levels of cAMP dependent proteins, increased levels of calmodulin and the appearance of oncomodulin (Swierenga et al., 1980).

C. Glutathione Metabolism

Glutathione (L-gamma-glutamyl-L-cysteinylglycine) is present in millimolar levels in virtually all animal cells and most plants and bacteria (Meister, 1983 and 1988; Mannervik, 1987). Reduced glutathione is the primary low molecular weight thiol responsible for maintaining

intracellular reducing conditions. Glutathione is involved in many facets of metabolism, including reduction of disulfides in proteins, synthesis of deoxynucleotides, processing of prostaglandins and leukotrienes, interorgan transport of cysteine and also acts as a coenzyme for several enzymes (Meister and Anderson, 1983). In addition, glutathione has been shown to be directly involved in the protection of cells from free radicals and the conjugation and detoxification of drugs (Meister and Anderson, 1983; Wolf et al., 1987). These functions have important ramifications in neoplastic tissues.

Glutathione is synthesized intracellularly by the action of two enzymes: gamma-glutamylcysteinyl synthase and glutathione synthase (FIGURE 6) (Meister and Anderson, 1983). Gamma-glutamylcysteinyl synthase is subject to feedback inhibition by reduced glutathione and hence glutathione levels are maintained at a constant value of ~10mM (Richman and Meister, 1975). The catabolism of GSH is brought about by gamma-glutamyl transpeptidase which transfers the gamma-glutamyl portion of the molecule to an acceptor such as cystine, glutamine, water or GSH itself. Glutathione is manufactured mainly by the liver and is transported across the cell membranes. Once GSH reaches the extracellular environment, it is free to interact with gamma-glutamyl transpeptidase, located on the external surface of the cellular membrane. Gamma-glutamyl transpeptidase action

FIGURE 6

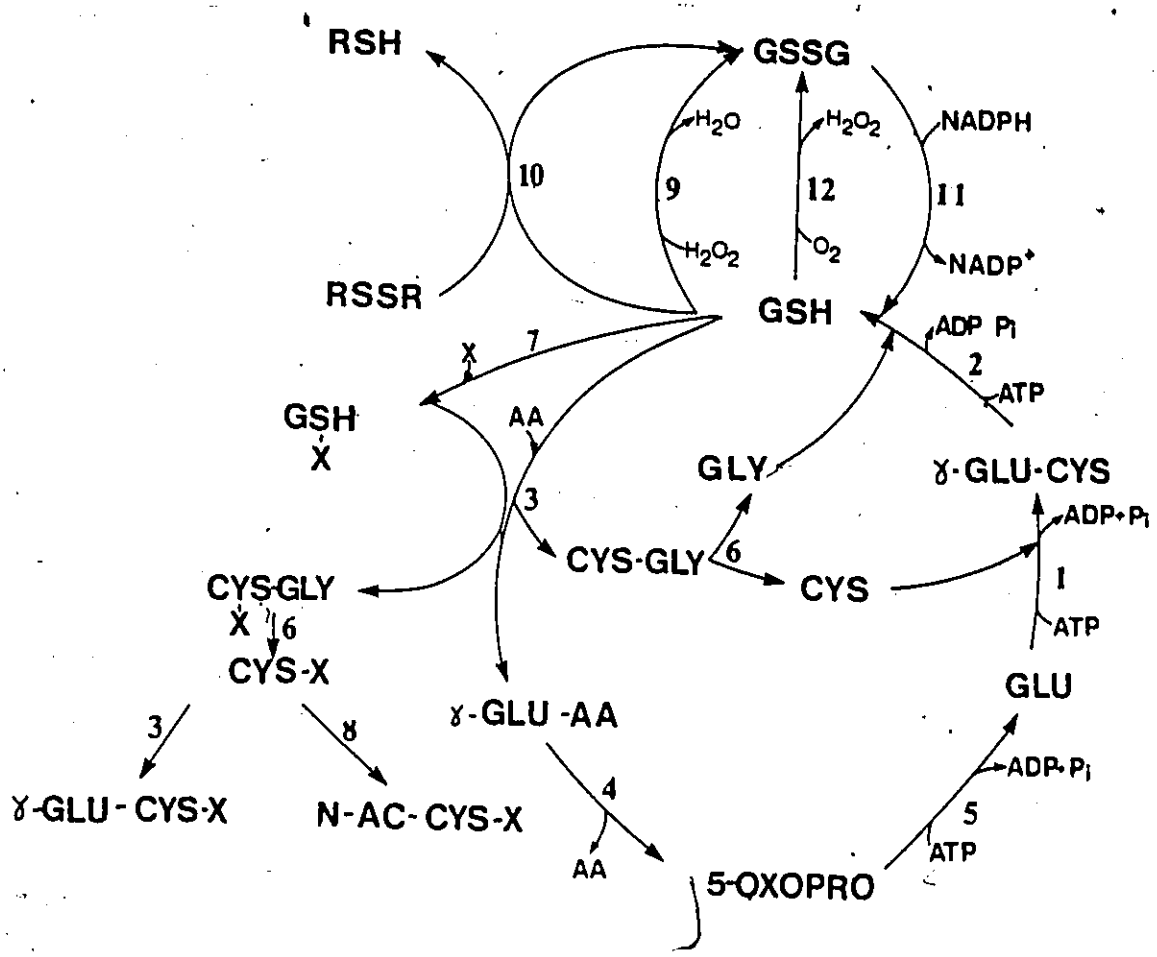


FIGURE 6: Glutathione Metabolic Pathway: Reaction 1: gamma-glutamylcysteinyl synthase; Reaction 2: glutathione synthase; Reaction 3: Glutamate transpeptidase; Reaction 4: gamma-glutamyl cyclotransferase; Reaction 5: 5-oxoprolinase; Reaction 6: dipeptidase; Reaction 7: GSH S-transferase; Reaction 8: N-acetylase; Reaction 9: GSH peroxidase; Reaction 10: transhydrogenase; Reaction 11: glutathione reductase; Reaction 12: oxidation of GSH by oxygen. (Meister and Anderson, 1983)

results in the formation of gamma-glutamyl amino acids which are then transported into the cell and converted by the action of gamma-glutamyl cyclotransferase to the corresponding free amino acid and 5-oxo-L-proline. 5-Oxo-prolinase then converts 5-oxo-proline to L-glutamate. The cysteinylglycine which is the second product of the transpeptidase is cleaved by the action of dipeptidase. These reactions are known as the glutathione cycle. This metabolic pathway is actually not a true cycle in that the reactions do not all occur within the same tissue; the synthesis of glutathione is performed mainly by the liver which then transports it out into the bloodstream for travel to other tissues which then metabolize it. The oxidation of reduced glutathione is brought about by the action of peroxidase or the interaction of GSH with disulfides via transhydrogenases and also occurs spontaneously in oxygen. The oxidized glutathione produced is toxic to the cell and is rapidly converted to the reduced form by the action of glutathione reductase.

In normal cells, reduced glutathione is present at a ratio of 300:1 to oxidized glutathione (Akerboom et al., 1982). This high ratio is maintained by the action of the ubiquitous enzyme, glutathione reductase. The catalytic mechanism of glutathione reductase is shown in FIGURE 7 (Pai and Schulz, 1983). The enzyme is a disulfide-linked dimer of two identical subunits of M_r 50000 and contains one FAD

FIGURE 7

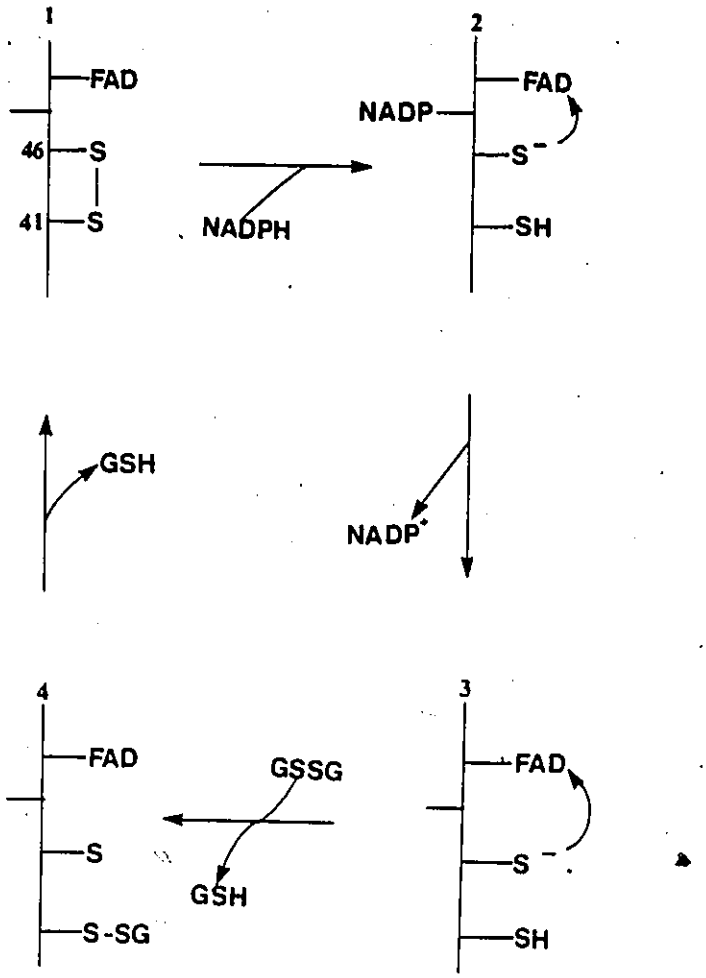


FIGURE 7: Catalytic Mechanism of Glutathione Reductase. The enzyme accepts two electrons from NADPH and transfers them via the FAD ring to the active site disulfide (cys 41 and 46). These two electrons are then donated to GSSG resulting in the reduction of GSSG to GSH and the concomitant oxidation of the active site back to the disulfide (Schulz et al., 1978; Pai and Schulz, 1983).

molecule per subunit. The reaction that is catalyzed is NADPH dependent and involves the transfer of reducing equivalents from NADPH via the FAD ring to the active site disulfide which then becomes reduced. This reduced form of the enzyme then converts the oxidized glutathione to the reduced form with concomittant oxidation of the active site thiols back to the disulfide. The complete primary and tertiary structure of the reductase have been determined (Schulz et al., 1978; Karplus et al., 1989; Karplus and Schulz, 1987). The active site to which the GSSG binds is located in the cleft formed where the two subunits join and is quite specific for the natural substrate (FIGURE 8).

Several reports have been published illustrating that the level of glutathione is elevated in neoplastic tissues compared to their normal counterparts (Russo et al., 1986; Arrick and Nathan, 1984). It has been determined that reduced glutathione acts as a control for its own biosynthesis via feedback inhibition of gamma-glutamylcysteinyl synthase, the first committed step of the glutathione biosynthetic pathway (Richman and Meister, 1975). This feedback inhibition appears to have been overruled in tumour cells resulting in increased levels of glutathione. The elevated levels of glutathione are postulated to afford increased resistivity to chemotherapeutic agents and radiation through detoxification of the drug itself or the reactive oxygen compounds produced (Russo et al., 1986; Crook

FIGURE 8

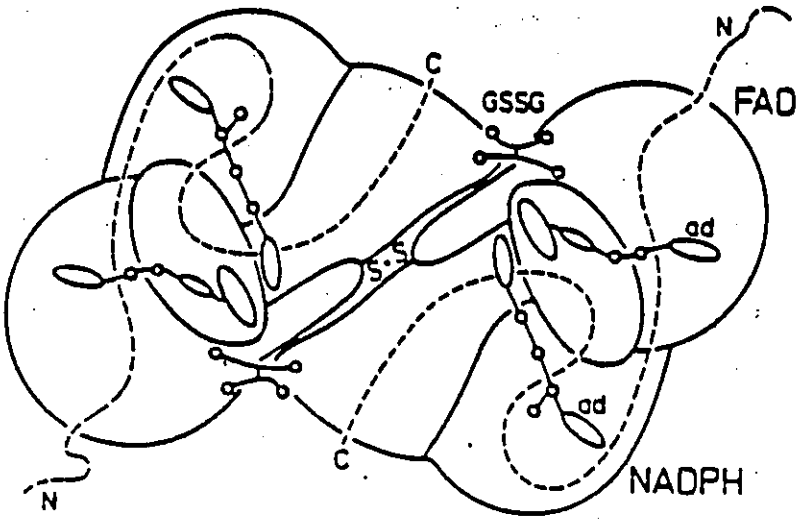


FIGURE 8: Tertiary Structure of Glutathione Reductase. Shown is a schematic diagram of dimeric GSSGRase. FAD and NADPH are illustrated bound to the enzyme. GSSG binds at the interface of the two subunits. The general course of the polypeptide chain is represented by the dashed line. (Reproduced from Pai and Schulz, 1983).

et al., 1986; Arrick and Nathan, 1984). In addition, the development of resistance to chemotherapeutic agents is also thought to be a result of increased levels of glutathione (Meister, 1985 and 1988; Wolf et al., 1987). The inhibition of gamma-glutamylcysteinyl synthase by buthionine sulfoximine results in decreased glutathione levels in both normal and neoplastic cells (Lee et al., 1987; Meister, 1985 and 1988). Tumour cells which were treated with this inhibitor have been shown to become more sensitive to chemotherapeutic agents and radiation, presumably as a result of decreased glutathione levels (Lee et al., 1987; Russo et al., 1986). Therefore it would appear that if one could selectively alter glutathione metabolism in tumour tissues compared to the host cells, antineoplastic treatment would be more selective, killing neoplastic tissue which had previously been depleted of glutathione. Normal cells, containing their normal complement of glutathione or perhaps excess through some chemical means would be left relatively unscathed by the treatment. Russo and coworkers have published some work in this area on the selective modulation of glutathione levels (Russo et al., 1986). They determined that selective elevation of human lung fibroblast cell glutathione levels could be achieved by treatment with 2-oxothiazolidine-4-carboxylate. This compound is an analog of 5-oxo-L-proline, where the 4-methylene group is replaced by a sulfur atom, and can act as a substrate for 5-oxoprolinase which converts it

to S-carboxy-L-cysteine which then breaks down spontaneously to L-cysteine (Meister, 1985). The elevation of glutathione levels observed in the normal cell line in vitro was selective in that the corresponding neoplastic cell, a human lung adenocarcinoma cell line, illustrated no alteration in glutathione levels. Therefore, it appears promising that one could be able to selectively protect normal cells prior to antineoplastic treatment by increasing their glutathione levels relative to neoplastic tissues.

A second difference in glutathione metabolism was observed with treatment with buthionine sulfoximine. Normal cells treated with this compound illustrated a faster rate of glutathione depletion than their neoplastic counterparts in vitro (Russo et al., 1986). Therefore, it would appear that subsequent to glutathione depletion with buthionine sulfoximine, antineoplastic agents which require cellular reduced glutathione for reduction, such as neocarzinostatin, would be more selective for tumour tissues where the depletion of glutathione is lesser (DeGraff et al., 1985).

The mechanism by which tumour cells maintain this higher level of total glutathione is unknown. It is possible that neoplastic tissues have an altered ratio of reduced to oxidized glutathione and in this way contain higher levels of total glutathione while leaving reduced glutathione levels constant to avoid problems with the negative feedback mechanism discussed previously. This would result in an

excess of oxidized glutathione compared to normal cells. This oxidized glutathione would then be converted upon the application of external metabolic stress, such as chemotherapeutic agents, by the action of glutathione reductase. Glutathione reductase is also involved in recycling oxidized glutathione produced by the action of glutathione peroxidase upon conversion of hydrogen peroxide to water, again decreasing the deleterious effects of antineoplastic treatment. In support of this argument, glutathione reductase has been shown to be an inducible enzyme in rat liver when the cells were treated with various compounds such as trans-stilbene oxide (Carlberg et al., 1981). Enzyme levels were found to increase to 250% of the control levels. This suggests that glutathione reductase levels are of importance for defense against toxic agents.

As a result of the above studies and others, it has been determined that glutathione levels are an integral part of the altered biological function of neoplastic tissues. Several neoplastic tissues which have illustrated increased levels of glutathione also express oncomodulin such as human lung adenocarcinomas (Russo et al., 1986; MacManus et al., 1984) and murine sarcomas (Lee et al., 1987; MacManus, 1981). Since oncomodulin is native to many neoplastic tissues, it would be very interesting if oncomodulin could in some way be involved in the maintenance of these altered glutathione levels.

8. Goals of the Present Study

Oncomodulin has been purified to homogeneity from rat Morris hepatomas by J.P.MacManus. The primary structure has been determined and illustrates extensive homology to the beta parvalbumin subclass of the Troponin C superfamily. However, some reports have surfaced indicating that oncomodulin is able to mimic the action of calmodulin in the activation of bovine and rat heart phosphodiesterase and also in the stimulation of DNA synthesis in Ca^{2+} deprived rat liver cells. To date, however, the concentrations necessary for activation of calmodulin dependent enzymes were too high to be of physiological relevance.

This study will center on the ability of oncomodulin to dimerize and the structural and functional consequences of the dimerization. Superficially, one can think of oncomodulin as half a calmodulin molecule; oncomodulin has two calcium binding loops whereas calmodulin has four. Therefore, oncomodulin dimer would more closely resemble calmodulin, having four calcium binding loops arranged in two domains joined by a central helical segment. The structural consequences will be determined by studying the ability of oncomodulin monomer and dimer to interact with melittin which is thought to be a model of the minimal structural requirements necessary for the calmodulin target

protein binding domain. The two forms of oncomodulin will then be compared with respect to their calmodulin like function; in order to determine this, two calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, will be tested for their relative activation by oncomodulin monomer and dimer.

In addition, oncomodulin monomer and dimer will be tested for their ability to modulate glutathione metabolism. This will be determined by studying the interaction of oncomodulin with glutathione reductase which has been shown to be an integral part of the mechanism by which neoplastic cells protect themselves from the lifethreatening effects of chemotherapy and radiation.

Oncomodulin is expressed only during development and again upon neoplastic transformation; this suggests that oncomodulin is in some way necessary to the viability of these systems. The elucidation of the role of oncomodulin in neoplastic tissues will perhaps shed some light on the altered control mechanisms which result in and exist upon neoplastic transformation.

MATERIALS

Material	Supplier
acrylamide	Sigma
ammonium persulfate	Sigma
bis-acrylamide	BDH
bovine brain	CDR Meat Packers
bovine heart	CDR Meat Packers
bovine serum albumin	Sigma
$CaCl_2$	Sigma
Calmodulin-Sepharose	Sigma
cAMP	Sigma

carp parvalbumin	Dr. L. Lee Dept. Chemistry and Biochemistry Univ. of Windsor Windsor, Ontario
Chelex 100	Biorad
cyanogen bromide	Aldrich
dansyl chloride	Sigma
DEAE Sephadex	Sigma
dithiothreitol	Sigma
DTNB	Sigma
EDTA	Sigma
EGTA	Sigma
FAD	Sigma

glutathione reductase (bovine intestinal mucosa) (Type VII; EC 1.6.4.2.)	Sigma
GSH	Sigma
GSSG	Sigma
HPLC Molecular Weight standards	Biorad
iodoacetamide	Sigma
lauryl sulfate	Sigma
magnesium acetate	Sigma
melittin (bee venom)	Sigma
2-mercaptoethanol	Sigma
NADPH	Sigma
5'-nucleotidase (Crotalus atrox venom)	Sigma

oncomodulin	Dr. J. P. MacManus Division of Biological Sciences National Research Council of Canada Ottawa, Ontario
Phenyl Sepharose	Sigma
PMSF	Sigma
pNPP	BDH
rabbit parvalbumin	Sigma
rat liver	Pel-Freez
rat parvalbumin	Dr. J. P. MacManus
SDS-PAGE Molecular Weight standards	Sigma
Sephadex G-25	Sigma
Sepharose 4B	Sigma
TEMED	Sigma

tris(hydroxymethyl) amino-methane	Sigma

APPARATUS

Absorbances were measured on a Shimadzu UV-160 Spectrophotometer equipped with a temperature block.

Fluorescence measurements were performed on a Shimadzu RF-540 Spectrofluorophotometer.

Electrophoresis was performed on a Bethesda Research Laboratories Vertical Gel Electrophoresis system.

All experiments were performed with distilled deionized water obtained from a Zenopure MEGA-90 water system unless otherwise stated.

Protein elution profiles were monitored by a Biorad UV Monitor Model 1306 connected to a Shimadzu C-R3A Chromatopac Integrator and a Pharmacia Fraction Collector FRAC-100.

HPSEC experiments were performed on a Biorad TSK-125 column (300 X 7.5mm) connected to a Biorad HPSEC Pump Model 1330.

All plots were performed on Sigmaplot Version 3.1.

METHODS

1. PROTEIN PURIFICATIONS

A. Calmodulin

Calmodulin was purified from bovine brain according to Sharma and Wang (Sharma & Wang, 1979). The partially thawed tissue was chopped fine and homogenized in 20mM Tris-HCl; 1mM $Mg(CH_3COO)_2$ pH 7.5 at 4°C (Buffer A) containing 10mM BME, PMSF and 1mM EGTA using a Waring blender. The homogenate was then centrifuged for 40 minutes at 4°C and ~2500g using an IEC PR-6000 centrifuge. The supernatent was filtered through glass wool and applied onto DEAE-Sephadex (9 x 20cm), equilibrated with the homogenization buffer. The column was then washed with 2L of Buffer A containing 10mM BME, PMSF, 0.1mM EGTA and 0.05M KCl. The CaM was then eluted with 1L of 0.5M KCl in the above buffer and the protein dialyzed overnight against 16L Buffer A containing PMSF and 0.1mM $CaCl_2$. The protein was then applied to ~100mLs of phenyl Sepharose (5 x 5cm), pre-equilibrated with Buffer A containing 0.05mM $CaCl_2$. The column was then washed with the same buffer containing 0.2M NaCl. The CaM was eluted with Buffer A containing 0.1mM EGTA and 0.2M NaCl. The protein was then dialyzed against distilled deionized water, lyophilized and stored at -20°C until needed.

B. Cyclic Nucleotide Phosphodiesterase

Cyclic nucleotide phosphodiesterase from bovine heart was purified according to Sharma and Wang (Sharma & Wang, 1979). The tissue was homogenized, centrifuged and applied to DEAE Sephadex in an identical fashion to the calmodulin purification. The enzyme was then eluted from DEAE Sephadex with 1L of Buffer A containing 10mM BME, 0.1mM EGTA, PMSF and 0.25M KCl. The eluted enzyme was then made 0.1mM in Ca^{2+}, stirred for one half hour and applied onto Affi-Blue Sepharose (9 x 10cm) in the same buffer. After washing, the enzyme was eluted by the application of the same buffer fortified with 1M NaCl. The PDE was then dialyzed overnight at 4°C against 16L of Buffer A containing 0.05mM $CaCl_2$, 10mM BME and PMSF. The enzyme was then applied onto calmodulin Sepharose (3 x 3cm), pre-equilibrated with Buffer A containing 0.1mM $CaCl_2$, 10mM BME, PMSF. The column was washed with the same buffer fortified with 0.2M KCl. The enzyme was then eluted with the addition of Buffer A containing 0.5mM EGTA, 10mM BME, PMSF and 0.2M KCl. The active fractions were pooled and dialyzed against 2L Buffer A containing 10mM BME and 40% glycerol.

C. Calcineurin Phosphatase

Bovine brain calcineurin was purified according to Sharma and coworkers (Sharma et al., 1983). The tissue was homogenized, centrifuged and applied to DEAE Sephadex as

above and eluted with Buffer A containing 0.13M KCl, 10mM BME, 0.1mM EGTA and PMSF. The enzyme was then made 0.5mM $CaCl_2$ and applied to calmodulin Sepharose and purified identically to the PDE purification procedure described above.

In order to ensure that the PDE and CaN preparations were not contaminated by CaM for the oncomodulin titrations, the enzymes were subjected to organomercurial Sepharose (OMS) chromatography. The enzymes were dialyzed against citrate buffer (0.1M) containing 20% glycerol, pH 6.8 to remove BME. The enzyme was then applied to OMS (3 x 3cm), pre-equilibrated with buffer (50mM Tris-HCl; 0.3M KCl; 0.1mM EGTA, pH 7.4). Any calmodulin present in the enzyme sample would not interact with the gel. The enzymes were then eluted by the addition of 10mM BME to the equilibration buffer. In the case of bovine brain CaN, 2M $MgCl_2$ was also added to the elution buffer.

In order to ensure that the enzymes were devoid of BME for the oncomodulin studies, the preparations were dialyzed against 0.1M citrate buffer containing 20% glycerol, pH 6.8.

D. Melittin

Melittin (obtained from Sigma) was further purified by CaM Sepharose affinity chromatography in the same manner as PDE and CaN.

E. Glutathione Reductase

Glutathione reductase was isolated from rat liver according to the method of Carlberg and Mannervik (1985). The rat livers (usually 25 per preparation) were thawed in 0.25M sucrose and a 20% (w/v) homogenization performed in the same solution. The homogenate was then centrifuged for 60 minutes at 10400g at 4 ° C. The supernatent was then filtered through glass wool and dialyzed for 8 hours against 20L 10mM sodium phosphate buffer pH 6.1 containing PMSF and 1mM EDTA. The sample was then applied to CM-cellulose (9 x 20cm) pre-equilibrated in the same buffer. The proteins were eluted by a linear concentration gradient of NaCl (0 to 0.2M) in 10mM sodium phosphate pH 6.1 containing 1mM EDTA (a total of 4L). The fractions were then assayed for GSSGRase activity and the active fractions pooled and concentrated to ~30mLs by ultrafiltration. The sample was then applied to a Sephadex G75 column (9 x 125cm) pre-equilibrated with 10mM sodium phosphate pH 6.7 containing 1mM EDTA and PMSF. The active fraction was pooled and applied to 2',5'-ADP Sepharose (20mLs) equilibrated with 50mM potassium phosphate pH 7.5 containing 1mM EDTA and 0.1mM BME. The column was then washed with 0.4M potassium phosphate pH 7.5 containing EDTA and BME as above. Glutathione reductase is then eluted by the application of 50mM potassium phosphate pH 7.5 containing 1mM EDTA, 0.1mM BME and 0.5mM NADPH. The enzyme (~20mLs) is then dialyzed overnight against 2L of 20mM Tris-

HCl pH 7.0 and concentrated by ultrafiltration. The enzyme is then stored at 4°C since freezing of GSSGRase results in a loss of activity.

2. AFFINITY COLUMN PREPARATIONS

A. Organomercurial Sepharose

Organomercurial Sepharose was prepared according to Sluyterman and Wijdenes (1970). 100mLs of Sepharose 4B was washed extensively and suspended in 100mLs of water. 25g of solid CNBr was added with stirring and the pH maintained at 11 by the addition of 2M NaOH for ~15 minutes. The suspension was then filtered and the activated gel washed with 1L of 0.1M $NaHCO_3$ pH 9.0. The gel was then resuspended in 200mLs of 10% DMSO at 4°C. 1.5g of p-aminophenylmercuric acetate (in DMSO) was then added and the mixture stirred overnight at 4 C. The suspension was then brought to room temperature, filtered and washed four times with 100mLs of 20% DMSO. This washing was then followed by 1L of 100mM EDTA pH 8.0 to chelate any remaining free mercurial. Finally the gel was treated with 100mLs of 1M glycine pH 8.0 in order to block any remaining active groups on the Sepharose.

B. Melittin Sepharose

Melittin Sepharose (Melex) was prepared according to Cox and coworkers (1985). Sepharose 4B was activated by CNBr treatment as for organomercurial Sepharose. After washing with 0.1M $NaHCO_3$ pH 9.0, the gel was suspended in 0.5M $NaHCO_3$ pH 8.3, 0.5M NaCl, 6M guanidine-HCl. Melittin was then added (1mg/mL gel) and the mixture stirred for 2 hours. The mixture was then washed with the same buffer and suspended in 1M glycine pH 8.0 as above.

C. Oncomodulin Sepharose

Oncomodulin Sepharose was prepared as follows. Sepharose 4B was CNBr activated as above. The gel was then incubated overnight at 4 ° C with oncomodulin (1mg/mL gel). Glycine was then added and allowed to react for 1 hour at room temperature. Before use, the column was washed with 100mM Tris-HCl pH 7.0; 10mM BME to ensure all the bound oncomodulin was in the reduced form.

3. PREPARATION OF MODIFIED PROTEINS

A. Dansyl Calmodulin

Dansyl calmodulin was prepared according to Kincaid and coworkers (1982). Bovine brain calmodulin was dialyzed overnight at 4 ° C against 10mM $NaHCO_3$ pH 10. A 1.5 fold

molar excess of dansyl chloride in acetone was then added with stirring. The protein was incubated for 90 minutes at 30 C and then dialyzed against distilled deionized water, lyophilized and stored at -20°C until needed.

B. S-carboxymido Labelled Oncomodulin

S-carboxymido labelled oncomodulin was prepared as follows. Reduced oncomodulin was passed through Sephadex G25 (1 x 28cm) to remove BME. Iodoacetamide (10 fold molar excess) at pH 8.5 was then added immediately and allowed to react for 1 hour at room temperature. The mixture was then run through Sephadex G25 a second time to remove excess iodoacetamide and the modified protein lyophilized and stored at -20°C.

S-carboxymethylation of ONC was performed in the same way, utilizing a 10 fold molar excess of iodoacetate, pH 8.5.

4. MELEX CAPACITY

DNS-CaM was utilized to determine the capacity of the Melex. Melex (~5mL; 1 x 1.5cm) was incubated for 15 minutes with an excess of DNS-CaM in 50mM Tris-HCl; 150mM KCl; 0.5mM $CaCl_2$; 4M urea pH 6.8. The unbound DNS-CaM was then removed by washing with the same buffer. The bound DNS-CaM was then

eluted with 50mM Tris-HCl; 600mM KCl; 1mM EGTA; 4M urea and quantitated.

5. AFFINITY CHROMATOGRAPHY ELUTION PROFILES

A. Melex Elution Profiles

The interaction of oncomodulin with Melex was determined as follows. Melex (~3mL; 1 x 1cm) was pre-equilibrated with 50mM Tris-HCl; 150mM KCl; 0.5mM $CaCl_2$; 4M urea, pH 6.8. Oncomodulin (reduced or oxidized) in 1mM $CaCl_2$ was then applied. After washing, the bound oncomodulin was eluted with 50mM Tris-HCl; 600mM KCl; 1mM EGTA; 4M urea pH 6.8.

B. Oncomodulin Sepharose Elution Profiles

Oncomodulin Sepharose (~5mL; 1.5 x 1cm) was pre-equilibrated with 100mM Tris-HCl; 1mM $CaCl_2$ pH 7.0. The enzyme (made 1mM $CaCl_2$) was then applied and the column washed with equilibrating buffer. Any bound enzyme was then eluted with 100mM Tris-HCl pH 7.0 containing 2mM EGTA.

C. Calmodulin Sepharose Elution Profiles

Calmodulin Sepharose (~5mL; 1.5 x 1cm) was pre-equilibrated with 100mM Tris-HCl; 1mM $CaCl_2$ pH 7.0. The enzyme (made 1mM Ca^{2+}) was then applied to the column and

the column washed with equilibrating buffer. Any bound enzyme was then eluted with 100mM Tris-HCl pH 7.0 containing 2mM EGTA.

6. PREPARATION OF REDUCED AND OXIDIZED ONCOMODULIN AND CARP PARVALBUMIN

Oxidized oncomodulin was prepared by incubating ONC in 50mM Tris-HCl; 150mM KCl pH 7.5 (Buffer B) containing 1mM $CaCl_2$ in the absence of reducing agents. The dimer was then separated from the monomer by OMS chromatography (1 x 2cm). The dimer which is disulfide linked passes directly through the column while the monomer remains bound to the OMS. The monomer was then eluted by the addition of 10mM BME to Buffer B.

Reduced and oxidized carp parvalbumin were prepared in a similar manner using 1mM EGTA in the incubation mixture to facilitate dimerization. OMS chromatography was then utilized as described for oncomodulin to separate the disulfide-linked dimer from the monomer.

7. ONCOMODULIN THIOL REACTIVITY

Reduced oncomodulin was separated from reducing agents

by Sephadex G-25 chromatography (1 x 28cm) in Buffer B containing either 1mM EGTA or $CaCl_2$. The protein was then transferred to a 1mL quartz cuvette and a 50 fold molar excess of DTNB was then added. The increase in absorbance was monitored at 412nm at 30°C.

8. RATE OF OXIDATION OF ONCOMODULIN

Reduced ONC (separated from BME by Sephadex G-25 chromatography) was allowed to oxidize at 30°C in the presence of 1mM EGTA or $CaCl_2$. Aliquots were removed with respect to time and reacted with DTNB (50 fold molar excess) in the presence of 0.1% SDS. The absorbance was then measured at 412nm.

9. STABILITY OF ONCOMODULIN DIMER

In order to determine the stability of ONC-dimer to 10mM GSH a time study was undertaken. ONC-dimer (~75ug) was incubated in 20mM Tris-HCl, 150mM KCl, pH 7.5 containing 10mM GSH and 1mM $CaCl_2$ at 30°C. Aliquots were removed at various time intervals and the pH of the sample lowered to ~4.0 by the addition of HCl. The samples were then subjected to 10% SDS-PAGE under nonreducing conditions

(Laemmli, 1970).

The stability of ONC-dimer to GSSGRase was determined in a similar manner. ONC-dimer (~75ug) was incubated with 1E-8M GSSGRase in the presence of calcium as above. Aliquots were removed with respect to time and the reduction terminated by the addition of HCl. The samples were then electrophoresed on 10% SDS-PAGE under nonreducing conditions.

10. ENZYME ASSAYS

A. Cyclic Nucleotide Phosphodiesterase

Cyclic nucleotide PDE was assayed according to Sharma and Wang (Sharma & Wang, 1979). The reaction mixture (0.8mL) contained 0.1mM $CaCl_2$, 0.3U 5'-nucleotidase, assay buffer (36mM Tris-HCl; 36mM imidazole; 4.5mM $Mg(OAc)_2$ pH 7.5), modulator protein and PDE. The reaction was then initiated with 100uL of 10mM cAMP and allowed to react for 30 minutes at 30 C. The reaction was terminated by the addition of 100uL 55% TCA. The inorganic phosphate was then quantitated by the addition of 1mL ammonium molybdate (0.55%) and 100uL of reducing agent (1-amino-2-naphthol-4-sulfonic acid). After development of the colour for 15 minutes at 30°C, the absorbances were measured at 660nm.

B. Calcineurin Phosphatase

Calcineurin activity was determined by the method of Pallen and Wang (Pallen & Wang, 1983). The assay mixture (500uL) contained Buffer B, 1mM Mn^{2+}, 1mM $CaCl_2$, CaM and modulator protein. The reaction was initiated by the addition of pNPP (10mM in cuvette). After 10 minutes incubation at 30° C the reaction was terminated by the addition of 500uL 10% K_2HPO_4. The amount of pNP produced was then determined at 405nm (molar extinction coefficient = 18000 $M^{-1}cm^{-1}$; Kincaid & Vaughan, 1986).

C. Glutathione Reductase

Glutathione reductase was assayed according to Carlberg and Mannervik (Carlberg & Mannervik, 1986). The reaction mixture contained 100mM Tris-HCl pH 7.0, GSSG, 1E-4M NADPH, 1mM $CaCl_2$ or EDTA and modulator protein as indicated (final volume 1mL). The reaction was then initiated by the addition of GSSGRase and allowed to proceed for 1 minute at 30°C. The reaction was terminated by the addition of 50uL 10% SDS and the absorbance measured at 340nm. The decrease in absorbance was then calculated by the subtraction of the absorbance of a blank to which no GSSG had been added.

11. MELEX COMPETITIVE BINDING ASSAY

The procedure utilized was a modification of the assay described by Cox and coworkers (Cox et al., 1985). 0.1uM DNS-CaM was added to Melex suspensions (with a capacity of binding 100% of the DNS-CaM) equilibrated with Buffer C (50mM Tris-HCl; 150mM KCl; 4M urea pH 6.8). containing 1mM $CaCl_2$. Varying amounts of competing proteins (ONC-dimer, ONC-monomer, CaM, TnC, cPV) were then added in a total volume of 1.2mLs in 1.5mL airfuge tubes. The assay tubes were then shaken for 30 minutes at 25°C and centrifuged in an Eppendorf centrifuge 5414. 800uL of supernatent was then carefully removed so as not to disturb the pelleted Melex. The sample was made 5mM in $CaCl_2$ to ensure that all the DNS-CaM was in the Ca^{2+} -saturated form. The amount of free DNS-CaM was then quantitated fluorometrically on a Shimadzu RF-450 spectrofluorometer (λex = 350nm; λ em = 505nm).

12. FLUORESCENCE MEASUREMENTS

All fluorescence measurements were performed at room temperature using a Shimadzu RF-540 spectrofluorometer. In order to detect the interaction of melittin with ONC-dimer, the tryptophan fluorescence of melittin was utilized. Samples were prepared in Buffer C containing 1mM EGTA or 1mM $CaCl_2$. Equimolar amounts of melittin and ONC-dimer (~1uM)

were added and incubated for ~15 minutes and their fluorescence spectra measured (λex = 290nm).

The fluorescence spectra of bovine intestinal mucosa and rat liver GSSGRase were also measured in order to detect the presence of endogenous NADPH. Solutions of known concentrations of NADPH and FAD were used as standards to correlate the amount of each cofactor present in the enzyme preparations. The fluorescence of NADPH was distinct from that of FAD in that the emission maximum of NADPH occurred at ~460nm whereas that for FAD was at ~520nm. The commercial enzyme preparation as well as the purified rat liver enzyme were found to contain up to a ten fold molar excess of NADPH.

13 ELECTROPHORESIS

The purity of all proteins was assessed by 10% SDS-PAGE using the Laemmli method (Laemmli, 1970).

SDS-PAGE was also used to determine the interaction of ONC-dimer with GSSGRase. Oxidized ONC (15ug) was incubated overnight (4°C) with GSSGRase (5E-10 moles) in the presence and absence of NADPH (4E-8 moles). ONC-dimer control samples were also incubated in the absence of GSSGRase with and without NADPH. The samples were then run the next day

on 10% SDS-PAGE under nonreducing conditions (Laemmli, 1970).

14. PROTEIN MONITORING

Unless otherwise stated, all protein elutions were monitored at 280nm on a Biorad UV Monitor Model 1306 connected to a Shimadzu C-R3A Chromatopac Integrator.

15. PROTEIN DETERMINATION

Protein determination was performed according to Bradford using BSA as a standard (Bradford, 1976).

16. CALCIUM TITRATIONS

Free Ca^{2+} concentrations in the calcium titrations were obtained by using EGTA buffering (Mutus et al., 1985) (APPENDIX 1). When it was necessary to achieve calcium concentrations below those of the Zenopure MEGA-90 distilled deionized water, Chelex-100 treatment was employed. The solution (water, buffer) was simply applied to the column

and collected. The resin is then regenerated with 1N HCl followed by H_2O, 1N NaOH and finally H_2O.

RESULTS

1. Oncomodulin Thiol Reactivity

Oncomodulin contains a single cysteine residue at position 18 of its primary structure. Since oncomodulin is a Ca^{2+}-binding modulatory protein as illustrated by MacManus (MacManus et al., 1984) and also by our own lab (Mutus et al., 1985a and 1985b), it was postulated that the accessibility of the thiol may differ in the Ca^{2+} saturated form of ONC compared to that in the absence of calcium. The reactivity of the thiol was probed using the thiol specific reagent DTNB in the presence and absence of Ca^{2+}. The cys-18 thiol appears to be more reactive toward DTNB in the Ca^{2+} saturated conformation (FIGURE 9). The second order rate constant, k_2, estimated from pseudo first order plots of the time courses in the presence of 1mM Ca^{2+} was 240+/-5 $M^{-1}min^{-1}$, ~10 fold larger than that obtained for the reaction in the absence of Ca^{2+} (performed in 1mM EGTA), 24+/-5 $M^{-1}min^{-1}$ (FIGURE 10).

2. Rate of Dimerization of Oncomodulin

The dimerization of oncomodulin via intermolecular

FIGURE 9

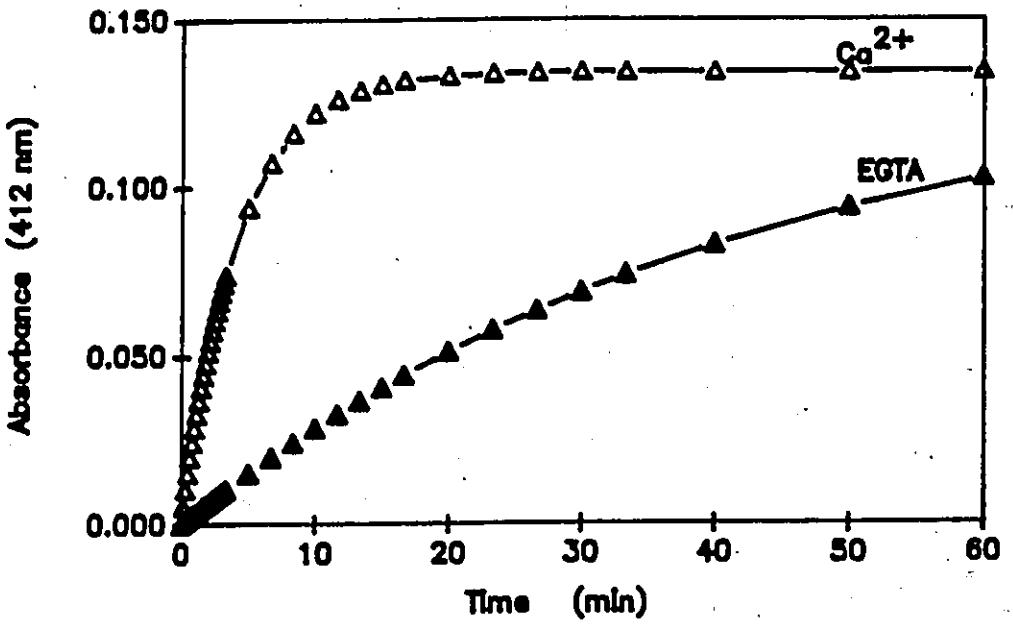


FIGURE 9: Reaction of Oncomodulin with DTNB: The reaction mixture consists of oncomodulin (1E-6M), DTNB (0.5mM), Ca^{2+} (1mM) (△) or EGTA (1mM) (▲) in Buffer A at 30 °C. Reaction is monitored at 412nm with respect to time.

FIGURE 10

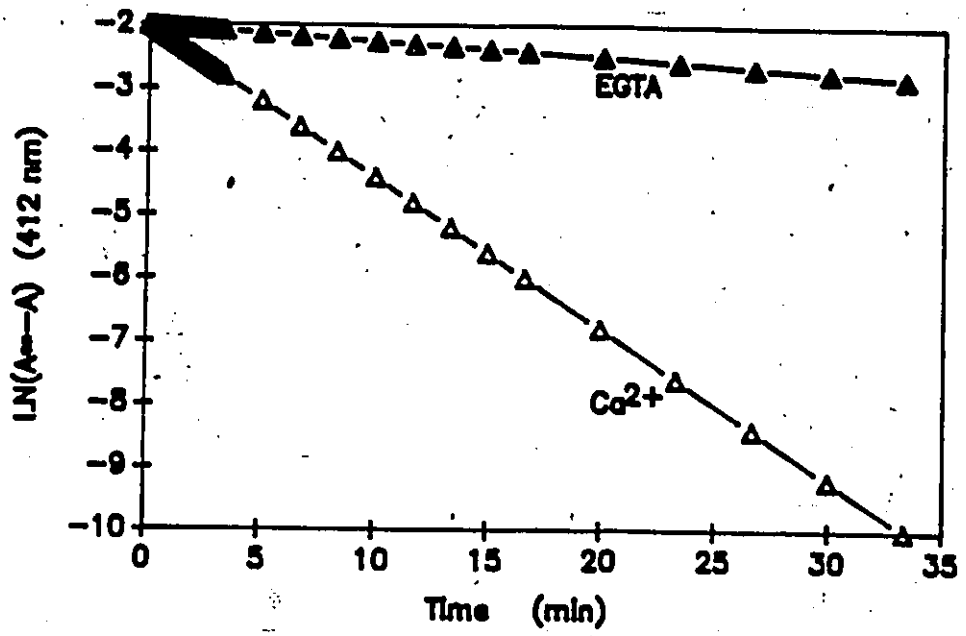


FIGURE 10: Pseudo First Order Plot of Reaction of Oncomodulin with DTNB. The results of FIGURE 9 were plotted according to the pseudo first order rate equation ($\ln(A_{\infty}-A) = -kt$). (Ca^{2+} △ ;EGTA ▲) The solid line represents the theoretical best fit of the data to the first order rate equation by the Simplex method (Noggle, 1985).

disulfide bond formation between cys-18 thiols was first suspected from the results observed on 10% SDS-PAGE (FIGURE 11). Typically, oncomodulin which was dialyzed and then electrophoresed in the presence of reducing agents ran at an anomalously high M_r of ~16000. However, if oncomodulin was run in the absence of reducing agents after being left at room temperature, the protein was converted to a larger M_r ~27000 band in a time dependent manner. This larger M_r band could then be converted to the lower M_r band by the introduction of reducing agents (20mM DTT) to the sample.

The rate of dimerization of oncomodulin <u>in vitro</u> was determined by monitoring the decrease in the concentration of free thiol with respect to time according to the following reaction:

(O)

2 ONCSH ----------> ONCS-SONC

The second order rate constant for the reaction was calculated as follows. Aliquots of oncomodulin were removed from the reaction mixture (containing either 1mM Ca^{2+} or EGTA) at 30 ° C with respect to time and titrated for free thiol content with a 50 fold molar excess of DTNB (FIGURE 12). The reactions with DTNB were performed in 0.1% SDS to ensure that intermolecular attraction between ONC molecules was not obscuring free thiols. ONC incubated in the presence of EGTA appeared not to dimerize to any appreciable

FIGURE 11

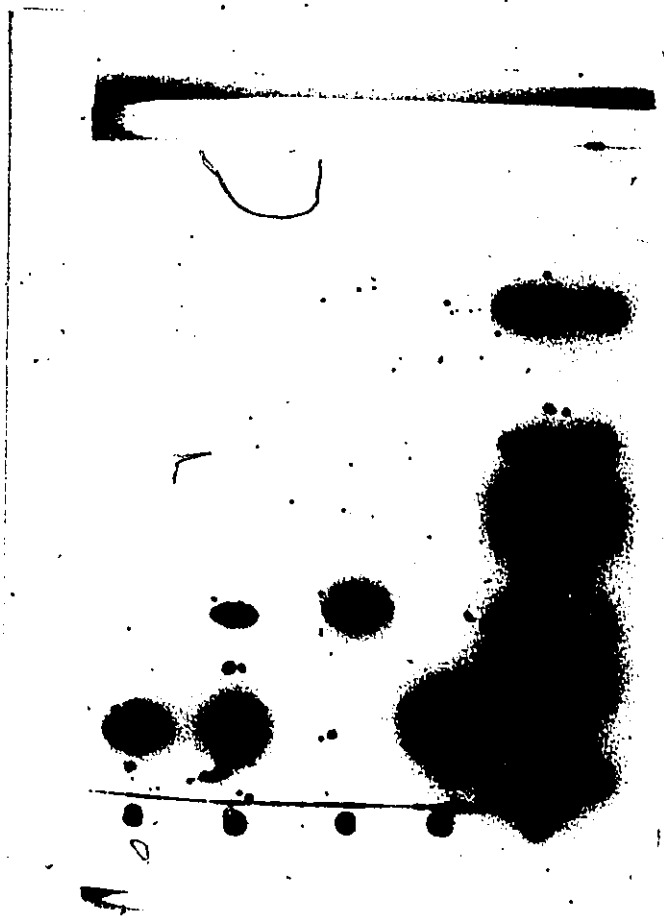


FIGURE 11: SDS-PAGE of Oncomodulin Forms. Lane a: oncomodulin left at room temperature t=0; Lane b: oncomodulin left at room temperature t=2 hours; Lane c: oncomodulin left at room temperature t=16 hours; Lane d: oncomodulin left at room temperature t=16 hours plus 20mM DTT; Lane e: molecular weight standards, bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20100) and lactalbumin (14200). All oncomodulin samples contain ~15ug.

FIGURE 12

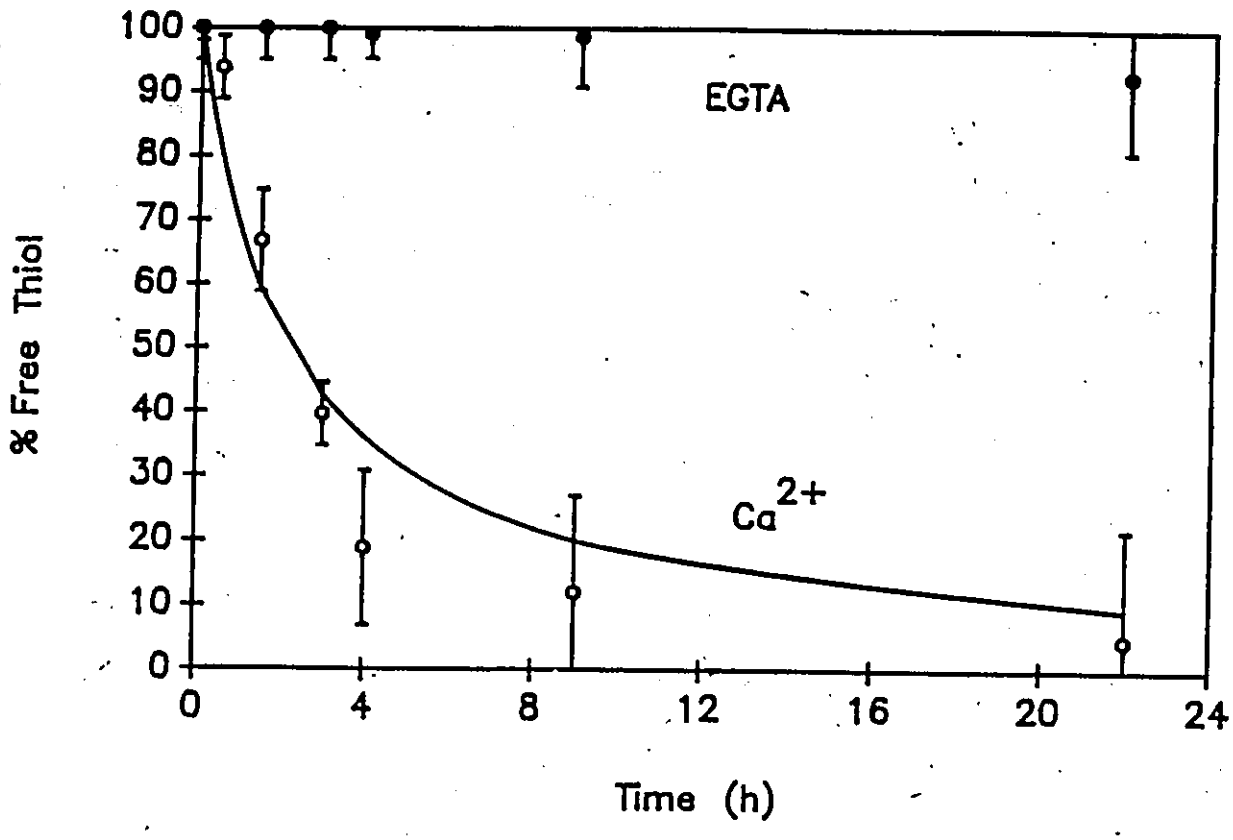


FIGURE 12: Time Course of the Oxidation of Oncomodulin Thiol. Oncomodulin (3.36E-5M), subsequent to the removal to DTT by Sephadex 625 chromatography, was incubated at 30°C. At various time intervals, samples (100uL) were removed and added to a cuvette which contained DTNB (0.5mM), SDS (0.1%) and Buffer A to a total volume of 1.0mL. The absorbance increase at 412nm was followed until the absorbance change was zero. The second order rate constant was obtained from a plot of the data according to the equation: $1/[ONC] = kt + 1/[ONC]_i$.

extent as there was no significant decrease in the thiol content for up to 4 hours. Even at the end of the 22 hour study, only ~5% of the sample was oxidized (FIGURE 12). In contrast, the oxidation of oncomodulin in the presence of Ca^{2+} was relatively rapid. The estimated second order rate constant was calculated to be 1500+/-230 $M^{-1}min^{-1}$. This is ~6 fold higher than that obtained for the reaction of oncomodulin and DTNB in 1mM Ca^{2+} possibly indicating that oncomodulin self associates via intermolecular electrostatic interactions prior to disulfide bond formation.

3. Stability of Oncomodulin Dimer to Reduced Glutathione

In order to determine the stability of ONC-dimer to intracellular reducing conditions, a time study on the effect of 10mM GSH on ONC-dimer was undertaken. ONC-dimer was incubated at 30°C in the presence of 1mM Ca^{2+} and 10mM GSH in 20mM Tris-HCl, 150mM KCl pH 7.5. At various time intervals, aliquots were removed and the pH lowered by the addition of 5uL 1M HCl. The samples were then run on 10% SDS-PAGE under nonreducing conditions (FIGURE 13). The results of the electrophoresis indicate that ONC-dimer was reduced to the monomer essentially simultaneously as no dimer was observed on the gel. However, the possibility that the reaction had not been terminated by the addition of

FIGURE 13

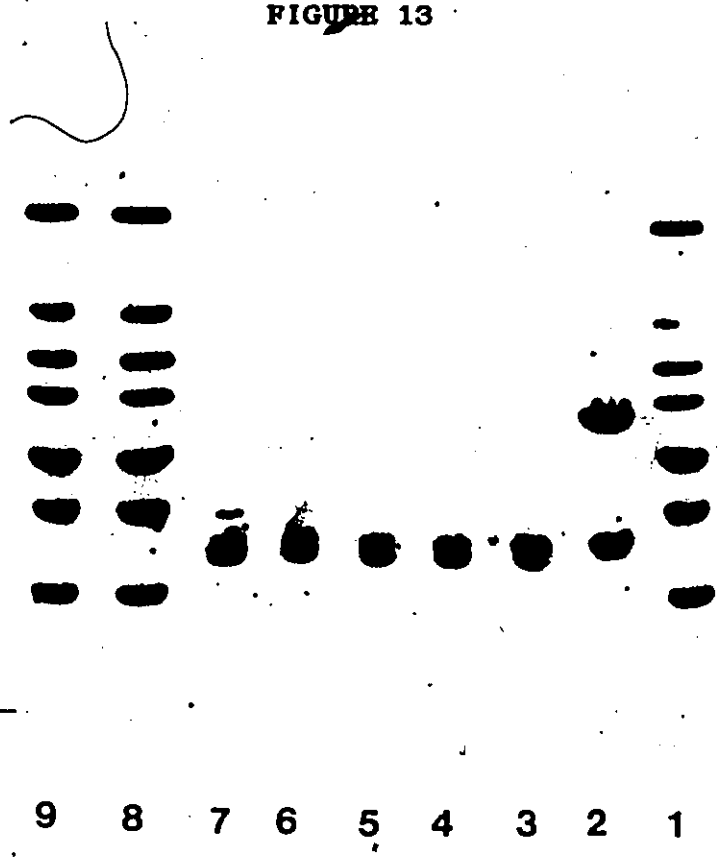


FIGURE 13: Stability of Oncomodulin Dimer to Reduced Glutathione. ONC-dimer was incubated at 30°C in 20mM Tris-HCl 150mM KCl pH 7.5 containing 10mM GSH and 1mM Ca^{2+}. Aliquots were removed with respect to time and the reduction stopped by lowering the pH. The samples were then electrophoresed on 10% SDS-PAGE under nonreducing conditions. Lane 1,8,9: molecular weight standards: bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20100) and lactalbumin (14200); Lane 2: ONC-dimer alone after acid treatment; Lane 3: ONC-dimer + GSH t="0; Lane 4: ONC-dimer + GSH t=5 minutes; Lane 5: ONC-dimer + GSH t=10 minutes; Lane 6: ONC-dimer + GSH t=20 minutes; Lane 7: ONC-dimer + GSH t=40 minutes.

acid cannot be ruled out; this would allow the conversion of ONC-dimer to the monomer upon the exposure of the disulfide by the addition of denaturing sample buffer.

4. Oncomodulin-Mellitin Interactions

Oncomodulin is known to contain 2 Ca^{2+} binding loops in contrast to calmodulin which contains four. Superficially, ONC-dimer would more closely resemble calmodulin in structure than ONC-monomer in that ONC-dimer would possess 4 Ca^{2+} binding loops. In addition, the two metal ion binding domains would be linked by a long peptide segment as in calmodulin which has been illustrated to be dumbbell shaped (Babu et al., 1985). In order to test this hypothesis, ONC-monomer and ONC-dimer were compared to calmodulin with respect to their secondary structural characteristics. The secondary structural properties of these proteins were studied by comparing their binding affinities for bee venom mellitin, which has been previously shown to possess the minimal structural requirements of the CaM binding domain of target proteins (Comte et al., 1983; Maulet & Cox, 1983; Cox et al., 1985; Caday & Steiner, 1986). Melittin is a 26 amino acid long protein which has two amphiphilic alpha helical segments joined by a bend. Melittin was shown by these workers to form a 1:1 complex with calmodulin in the

presence of calcium with a K_d in the nanomolar range. This is similar to the reported dissociation constants for calmodulin with its target proteins. In addition, the interaction of melittin with calmodulin was studied in the presence of 4M urea further illustrating the strength of the binding. Hence, if a protein is capable of binding to melittin with similar affinities as calmodulin, these proteins would also likely possess the secondary structural features of the target protein binding domain of calmodulin.

The first method utilized to determine the interaction of oncomodulin with melittin was the binding of oncomodulin to a melittin-Sepharose column (FIGURE 14). These experiments were performed in the presence of 4M urea in order to ensure that low affinity nonspecific binding (previously seen between calmodulin and melittin) did not occur. The elution profiles obtained with ONC-dimer indicate that nearly all of the ONC-dimer (~90%) bound to the melittin-Sepharose in the presence of 1mM Ca^{2+}. The adsorbed protein could then be eluted from the gel by chelation of the Ca^{2+} with EGTA. In contrast, ~75% of the ONC-monomer applied to the gel was not retained. The small amount of protein which was eluted by the application of EGTA is thought to represent the fraction of oncomodulin which spontaneously dimerized during the chromatography.

Additional evidence for the interaction of ONC-dimer with melittin was obtained by monitoring the changes in the

FIGURE 14

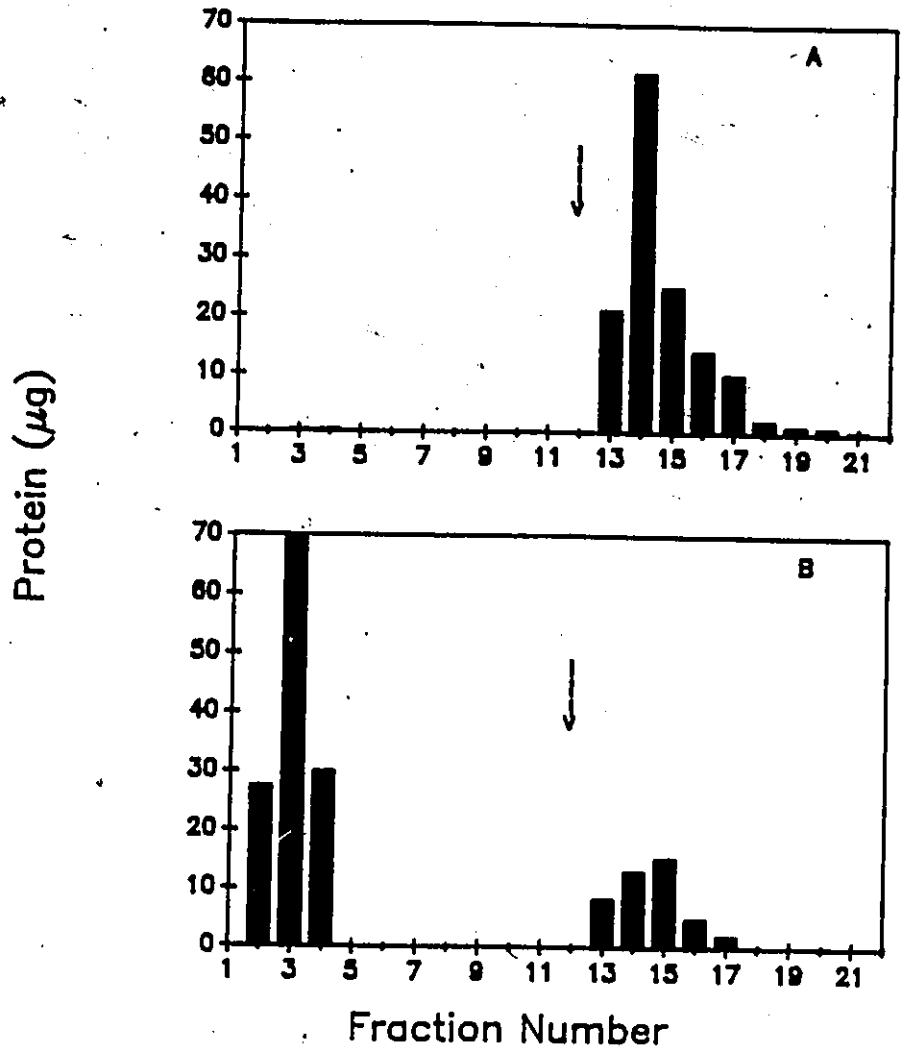


FIGURE 14: Melittin Sepharose Elution Profiles of Oncomodulin Monomer and Dimer. Panel A (ONC-dimer; 170ug) or Panel B (ONC-monomer; 180ug) was added to 0.5mL of melittin Sepharose gel suspension in Buffer A containing Ca^{2+} (0.1mM) and urea (4M) and placed on a horizontal shaker for 30 minutes. The gel was then packed into a column (0.5 x 5cm) and washed with the same buffer. At the point indicated by the arrow, the buffer was changed to Buffer A containing EGTA (0.1mM) and urea (4M) to elute the bound protein.

intrinsic tryptophan fluorescence of melittin (FIGURE 15). The emission spectrum of melittin was unaltered by the addition of ONC-dimer in the presence of 1mM EGTA. In the presence of 1mM Ca^{2+}, the emission spectrum of melittin was enhanced and blue shifted. These spectral changes are comparable to those obtained by others for melittin calmodulin and melittin S-100b complexes (Maulet & Cox, 1983; Baudier et al., 1987).

The previous work done by Cox and coworkers to study the interaction of calmodulin with melittin employed the use of melittin conjugated to Sepharose (which they termed Melex) (Cox et al., 1985). Their study involved the use of a competitive binding assay where sufficient radiolabelled calmodulin was added to a Melex suspension in 1mM Ca^{2+} and 4M urea to saturate the gel. Increasing amounts of non-radiolabelled calmodulin were then added and after incubation, the supernatent was removed and counted. The displacement curves obtained then yielded a K_d value for the dissociation of the melittin calmodulin complex. For our purposes, the procedure was modified by using a fluorescent labelled calmodulin, made by reacting dansyl chloride with calmodulin. The dansylcalmodulin has been shown to be functionally indistinguishable from the native protein since dansylcalmodulin was capable of activating bovine heart phosphodiesterase to the same extent and with the same $K_{activity}$ as native calmodulin (Kincaid et al., 1982). In

FIGURE 15

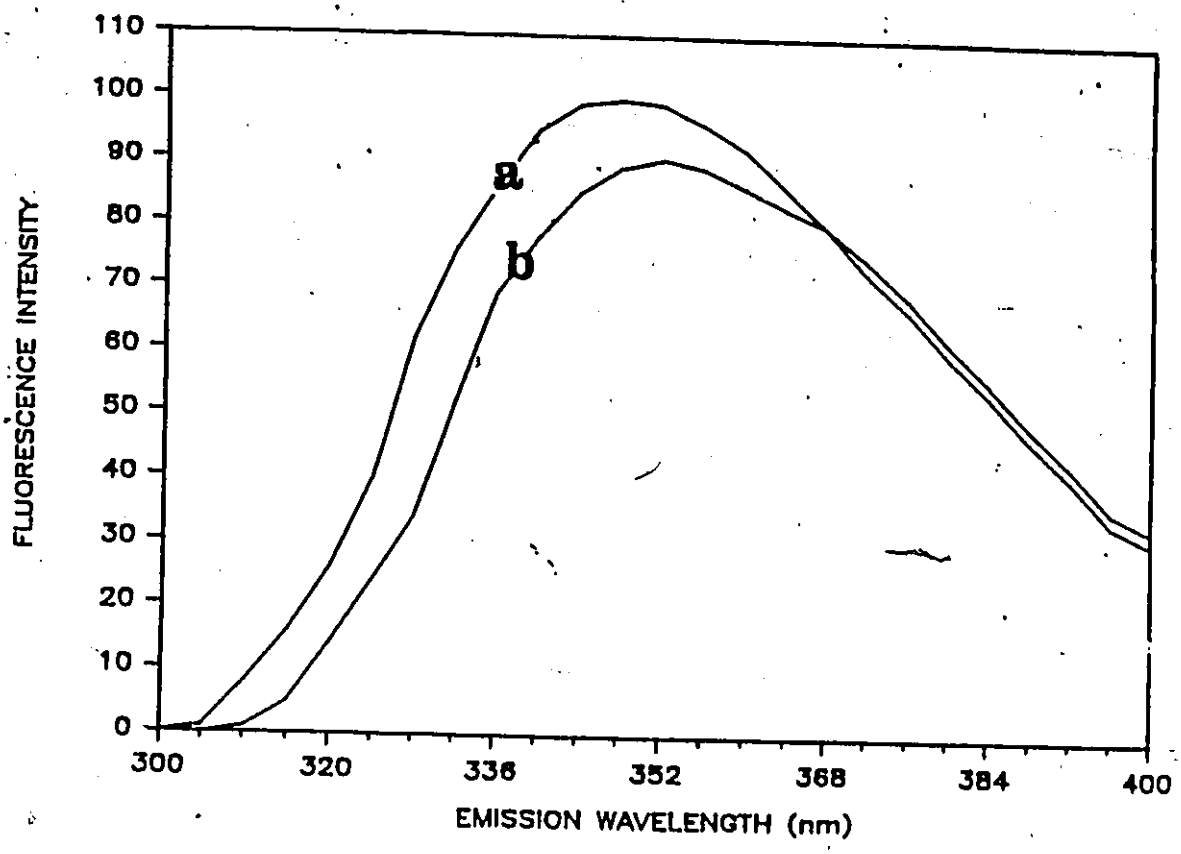


FIGURE 15: Fluorescence Spectra of Melittin in the Presence and Absence of ONC-dimer. Melittin (1E-6M) in Buffer A containing Ca^{2+} (1.0mM) and urea (4M) plus ONC-dimer (1E-6M) (A) and melittin (1E-6M) alone (B). The excitation wavelength was 325nm.

these experiments, sufficient DNS-calmodulin (0.1umol/L) was incubated with Melex to saturate the gel in 1mM Ca^{2+} and 4M urea. Increasing amounts of competing protein (calmodulin, ONC-dimer, ONC-monomer, TnC, carp parvalbumin) were then added and the mixture incubated for 30 minutes. The fluorescence of the supernatent was then determined as a percentage of the total DNS-calmodulin added. The equilibria describing the competitive binding are shown in FIGURE 16 where DNS-calmodulin and the competing protein, Comp, bind to Melex with the dissociation constants K_c and K_{comp}, respectively. Therefore, the fraction of DNS-calmodulin bound can be determined to be:

$$f_b=[DNS\text{-}CaM]/\{[DNS\text{-}CaM]+K_c(1+[Comp]/K_{comp})\}$$

(for derivation see Appendix 2). As a result, in the absence of competing proteins, at dansylcalmodulin concentrations ~100 fold higher than K_c, nearly all the dansylcalmodulin will be bound. In the case where the competing protein has the same affinity for Melex, such that $K_c=K_{comp}$, 50% of the bound DNS-calmodulin will be displaced when the concentration of competing protein equals that of DNS-CaM. This is what was observed when native calmodulin was added to the assay (FIGURE 17): when an equal amount of native calmodulin to labelled protein (1E-7M) was added to the assay, ~50% of the DNS-CaM had been displaced. This

FIGURE 16

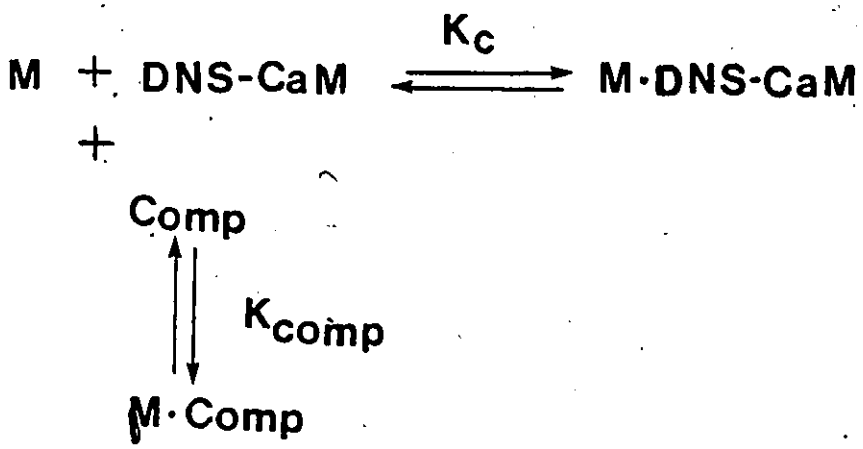


FIGURE 16: Equilibria of Competitive Binding Assay. M: Melex; Comp: competing protein. K_c and K_{comp} are the equilibrium constants for the illustrated reactions. Determination of fraction DNS-CaM is outlined in Appendix 2.

FIGURE 17

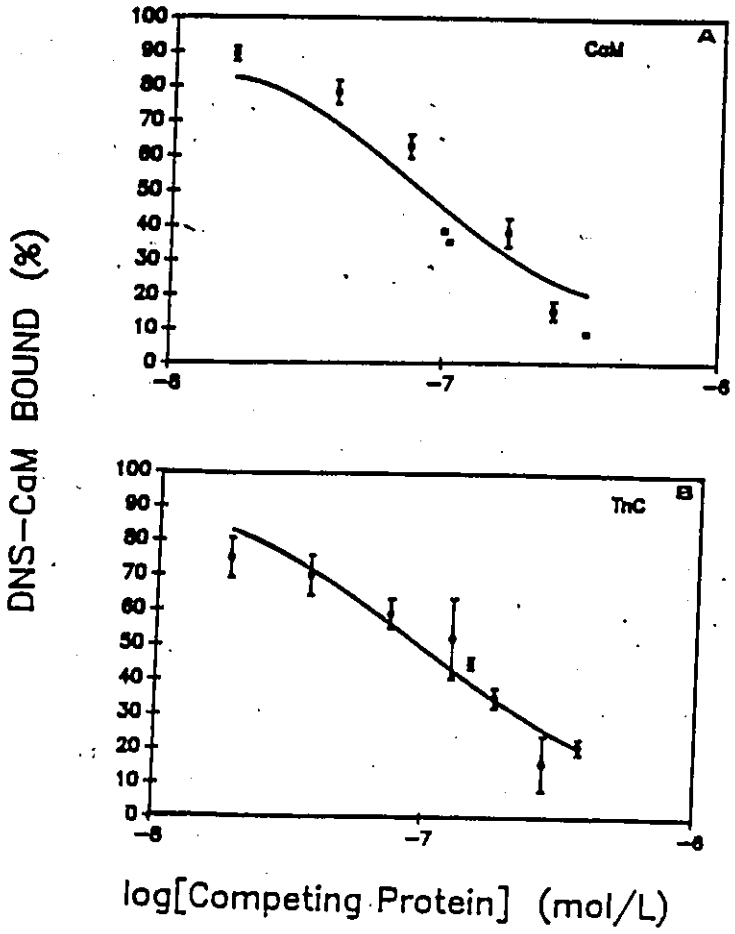


FIGURE 17: DNS-Calmodulin Displacement Assay in Presence of Calmodulin and Troponin C. DNS-CaM (1E-7M) was added to Helex suspension (0.4mL) (with sufficient gel to bind 100% of the DNS-CaM) in Buffer A containing Ca^{2+} (0.1mM) and 4M urea. Increasing amounts of competing proteins were then added and the mixture was then incubated for 30 minutes. The tubes were centrifuged and 0.8mL of supernatant was withdrawn and quantitated fluorometrically (λex = 350nm; λem = 505nm) for the amount of free DNS-CaM. Fraction bound was then calculated according to equations of FIGURE 16. The solid lines represent the theoretical best fit of the data by the Simplex method (Noggle, 1985). The data represent the average of three determinations. The error bars represent the standard error.

illustrates that dansylcalmodulin had the same affinity as the native protein for Melex. A fit of the displacement data by the simplex method (Noggle, 1985) yielded a K_{comp} for native calmodulin of ~0.6nM, in agreement with previously reported values (Comte et al., 1983).

The displacement profile which was obtained in the presence of increasing amounts of Troponin C was nearly superimposable on that of calmodulin yielding a K_{comp} of 0.9nM (FIGURE 17). Previous reports have also shown that Troponin C forms a Ca^{2+} dependent 1:1 high affinity complex with melittin (Steiner and Norris, 1987). This result was not surprising since it has been shown that Troponin C can substitiute for calmodulin in the activation of some target enzymes (Marcum et al., 1978; Picton et al., 1980). Carp beta parvalbumin, at the other extreme, was unable to displace dansylcalmodulin to any appreciable extent even at concentrations ten fold higher than calmodulin (FIGURE 18). This indicates that carp parvalbumin does not interact with mellitin with high affinity as was previously shown (Comte et al., 1983).

The displacement profiles obtained in the presence of ONC-dimer and ONC-monomer were dramatically different from each other (FIGURE 18). At a concentration of ONC-dimer equal to that of DNS-calmodulin, ~50% of the labelled calmodulin was displaced giving a K_{comp} of 1.1nM. On the other hand, ONC-monomer at the same concentration resulted

FIGURE 18

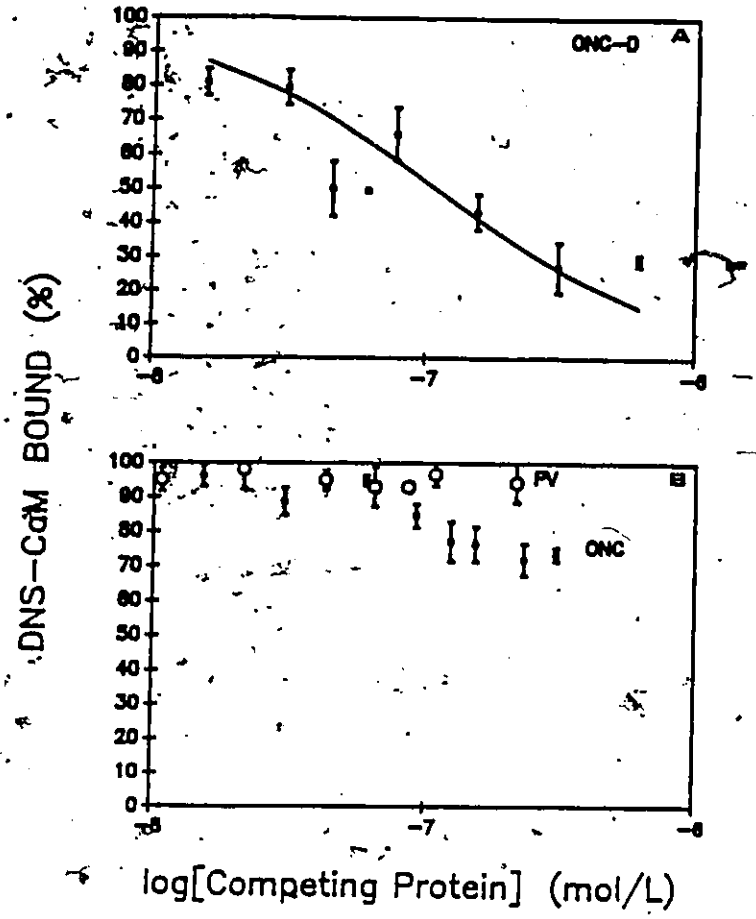


FIGURE 18: DNS-Calmodulin Displacement Assay in the Presence of Carp Parvalbumin, ONC-dimer and ONC-monomer. DNS-CaM (1E-7M) was added to Melex suspension (0.4mL) (with sufficient gel to bind 100% of the DNS-CaM) in Buffer A containing Ca^{2+} (0.1mM) and 4M urea. Increasing amounts of competing proteins were then added and the mixture was then incubated for 30 minutes. The tubes were centrifuged and 0.8mL of supernatant was withdrawn and quantitated fluorometrically (λex = 350nm; λem = 505nm) for the amount of free DNS-CaM. Fraction bound was then calculated according to equations of FIGURE 16. The solid lines represent the theoretical best fit of the data by the Simplex method (Noggle, 1985). The data represent the average of three determinations. The error bars represent the standard error.

in only ~15% of the dansylcalmodulin being displaced. Although insufficient displacement occurred to accurately fit the data to a displacement curve, the K_{comp} was estimated to be >1E-6M. These results suggest that the high affinity melittin binding domain on ONC-dimer is formed only upon dimerization and is not present on the reduced monomer. If this melittin binding domain is similar to that of the calmodulin target protein binding domain as has been suggested by the K_{comp} obtained in the presence of ONC-dimer, then ONC-dimer should also be able to interact with target proteins in the same concentration range as calmodulin whereas ONC-monomer should not.

5. Effect of ONC-dimer and ONC-monomer on Calmodulin Target Enzymes

In order to test whether ONC-dimer is more calmodulin like than monomer, two calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, were tested with respect to ONC-dimer and ONC-monomer dose-dependent effects on their catalytic activity. The activity of PDE as a function of ONC-dimer, ONC-monomer and CaM concentration is illustrated in FIGURE 19. ONC-dimer was capable of activating bovine heart PDE in a Ca^{2+} dependent manner to ~90% of that observed with calmodulin and gave a

FIGURE 19

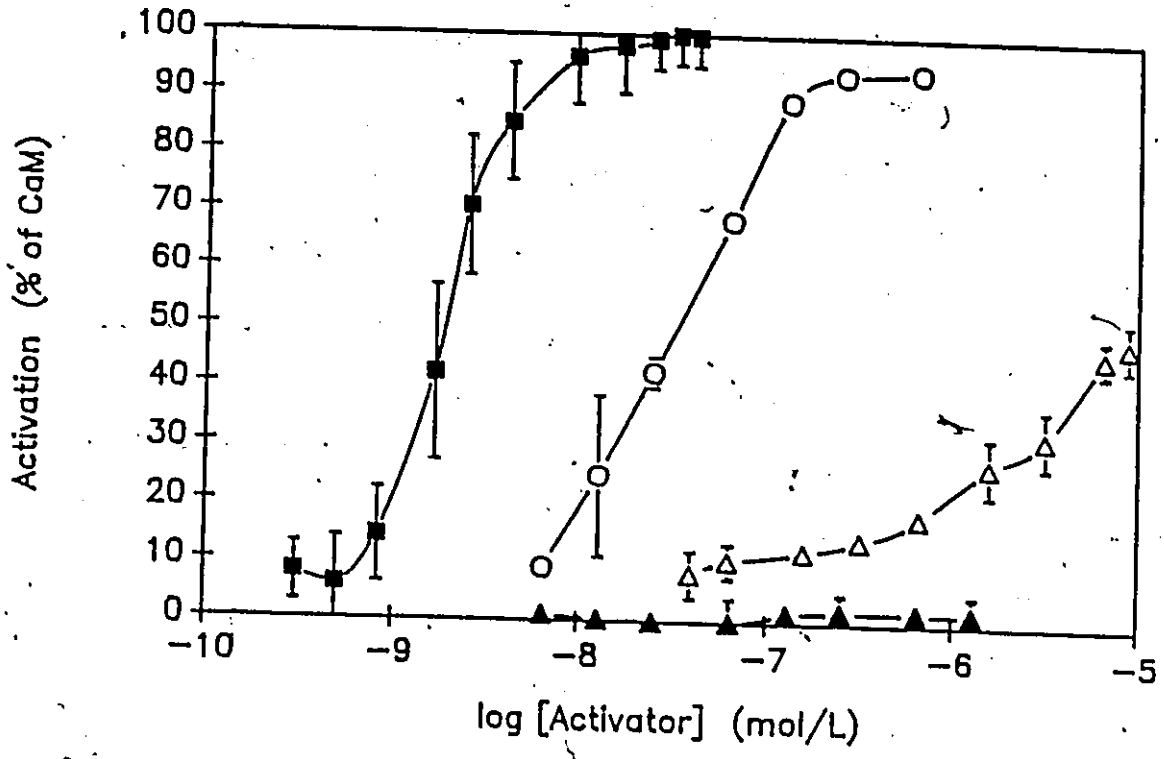


FIGURE 19: Titration of Bovine Heart Phosphodiesterase. Bovine heart phosphodiesterase activity was determined in the presence of increasing amounts of calmodulin (■), ONC-dimer (○), ONC-monomer (△) and S-carboxymethyl-ONC (▲). The PDE concentration was 0.1nM. Activation is defined as the activity at a given concentration of activator divided by the amount of activity produced by excess amounts of calmodulin, expressed as a percentage. PDE was activated 5.5 and 5 fold by calmodulin and ONC-dimer, respectively. The data points represent the average of four determinations. The error bars represent the standard error.

$K_{activation}$ of 63nM compared to 2nM for calmodulin. ONC-monomer also activated bovine heart PDE but with a much higher $K_{activation}$ of ~10uM. However, if ONC-monomer was S-carboxymethylated with iodoacetate, no activation of bovine heart PDE was observed. This suggests one of two things is occurring. The ONC-monomer will be dimerizing during the PDE assay which takes 30 minutes incubation time and hence the activation of PDE in the presence of ONC-monomer may be due to the amount of ONC-dimer formed. Blocking of the thiol does not allow this oxidation to occur. Alternatively, the activation of PDE by ONC-monomer may require the free thiol at cys-18 which would be abolished by the chemical modification with iodoacetate.

In the case of bovine brain calcineurin, only ONC-dimer was capable of Ca^{2+} dependent activation with a K_d of 1nM compared to 0.1nM for calmodulin (FIGURE 20). The K_d value obtained for calmodulin is in agreement with the reported value by Hubbard and Klee (Hubbard & Klee, 1987). ONC-monomer and S-carboxymethylated monomer were both unable to activate calcineurin.

It is evident from these results that the melittin binding domain of ONC-dimer has similar features to the calmodulin target protein binding domain in that activation of both these calmodulin dependent enzymes occurred in a similar range to that for calmodulin itself. This site of interaction is only formed upon dimerization of oncomodulin

FIGURE 20

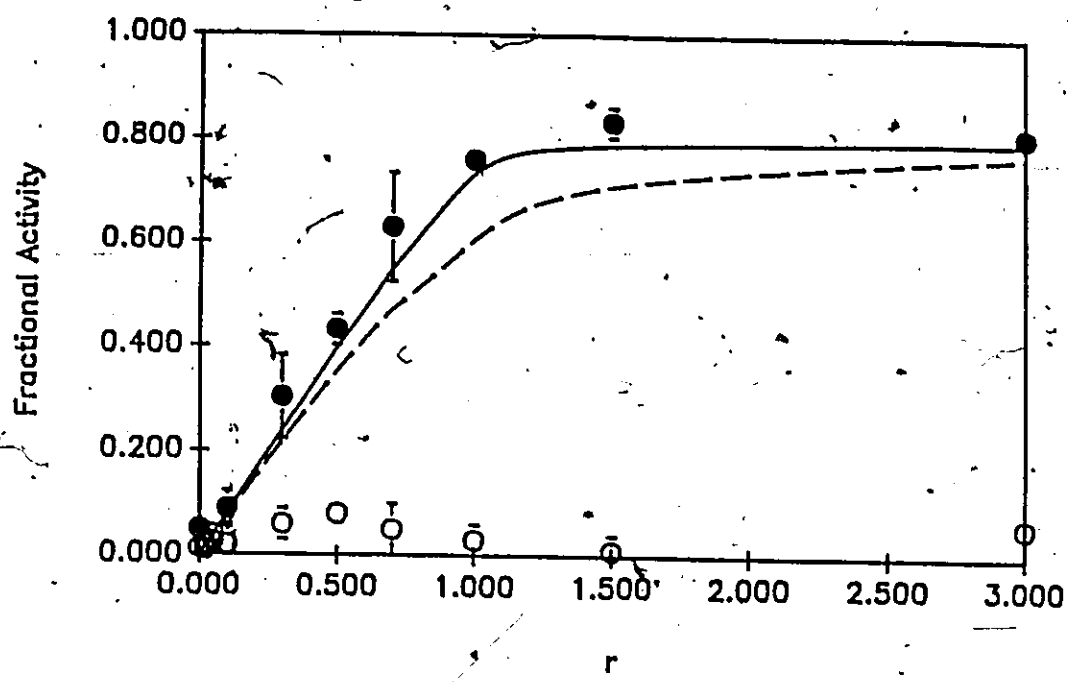


FIGURE 20: Titration of Calcineurin Activaty with ONC-monomer and ONC-dimer. Fractional activity is expressed as the activity at a given concentration of ONC-dimer (●) or ONC-monomer (○) divided by the activation observed in the presence of saturating amounts of calmodulin. CaN was activated 4.3 and 3.5 fold by calmodulin and ONC-dimer, respectively. The calcineurin concentration was 1E-7M. The solid line represents the theoretical best fit of the data to the Richards and Vithayathil (1985) equation ($a = 1/2(r + 1 + K' - \sqrt{[(r + 1 + K')^2 - 4r]^{1/2}}$) by the Simplex method (Noggle, 1985), where $r = [ONC]/[CaN]$, a = activity as a fraction of highest obtained and $K' = 1/K_d$. The K_d between calcineurin and ONC-dimer was calculated to be 1nM. The dashed line represents the theoretical line calculated for a K_d of 10nM. The data points are the averages of four determinations. The error bars represent the standard error.

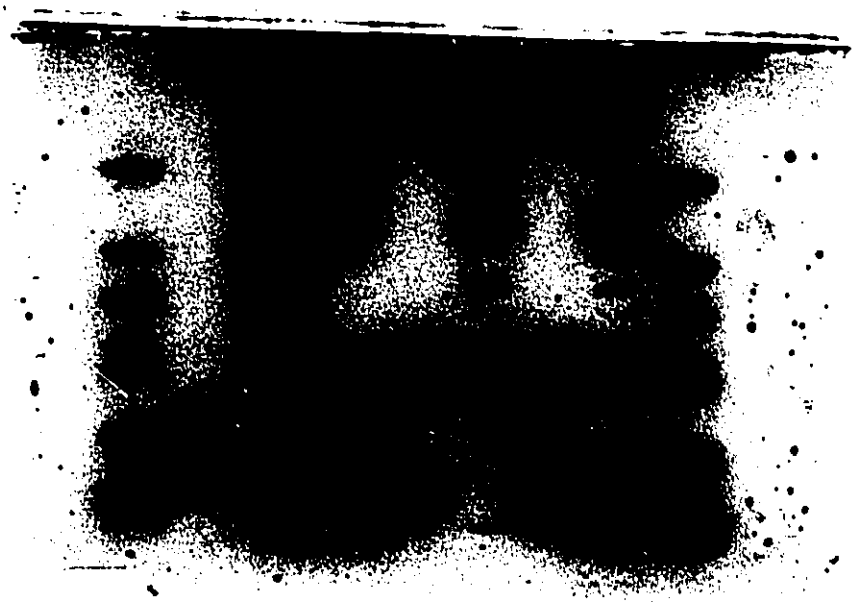
since oncomodulin monomer was unable to affect these enzymes in the same concentration range.

6. Interaction of Oncomodulin-Dimer with Glutathione Reductase

The interaction of oxidized oncomodulin with glutathione reductase was observed utilizing 10% SDS-PAGE under nonreducing conditions (FIGURE 21). ONC-dimer (M_r ~23400) was incubated overnight with glutathione reductase (BIM) in the presence and absence of NADPH. This incubation resulted in the conversion of oncomodulin dimer to the reduced monomer (M_r ~11700). ONC-dimer which was incubated alone or in the presence of only NADPH remained oxidized throughout the incubation. This indicates that glutathione reductase was necessary to effect the reduction of ONC-dimer and was in fact using ONC-dimer as a substrate.

A time study on the reduction of ONC-dimer by GSSGRase was also performed. ONC-dimer was incubated with 1E-8M GSSGRase in 1mM Ca^{2+} at 30 °C and aliquots were removed at various time intervals. The reaction was stopped by the addition of HCl and the samples were then subjected to 10% SDS-PAGE under nonreducing conditions (FIGURE 22). The results of the gel indicate that the reduction of ONC-dimer was essentially complete after five minutes.

FIGURE 21



1 2 3 4 5 6 7 8 9

FIGURE 21: SDS-PAGE Analysis of the Interaction of ONC-dimer with Glutathione Reductase. All samples were incubated overnight at 4 C before running under nonreducing conditions (Laemmli, 1970). Lane 1, 3, 9: molecular weight standards: bovine serum albumin (66200), ovalbumin (42700), glyceraldehyde 3-phosphate dehydrogenase (35000), carbonic anhydrase (28000), soybean trypsin inhibitor (20100) and lysozyme (14300); Lane 2: calmodulin; Lane 4: ONC-dimer (1.1E-9moles) incubated in the presence of GSSGRase (1.1E-9moles); Lane 5: ONC-dimer; Lane 6: GSSGRase; Lane 7: ONC-dimer plus NADPH (4E-8moles); Lane 8: ONC-dimer incubated with GSSGRase and NADPH.

FIGURE 22

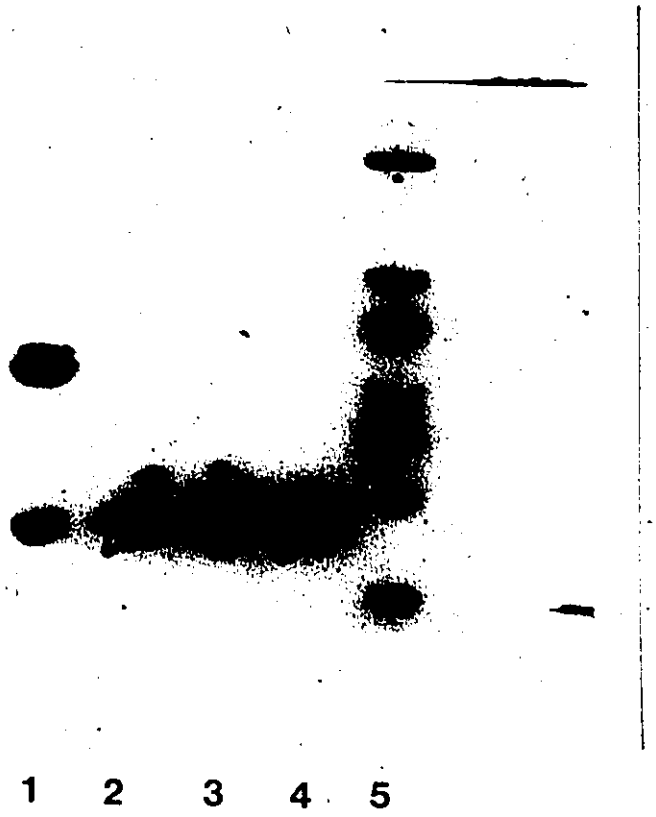


FIGURE 22: Time Study of the Interaction of ONC-dimer with Glutathione Reductase. ONC-dimer was incubated with 1E-8M GSSGRase in 1mM Ca^{2+} at 30°C. Aliquots were removed with respect to time and the reaction stopped by lowering the pH. Samples were then electrophoresed on 10% SDS-PAGE under nonreducing conditions. Lane 1: ONC-dimer following acid treatment; Lane 2: ONC-dimer +- GSSGRase t=5 minutes; Lane 3: ONC-dimer + GSSGRase t=15 minutes; Lane 4: ONC-dimer + GSSGRase t=30 minutes; Lane 5: molecular weight standards: bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), soybean trypsin inhibitor (20100) and lactalbumin (14200).

The known mechanism of GSSGRase requires the binding and oxidation of NADPH prior to disulfide reduction (Pai and Schulz, 1983). Therefore the incubation of GSSGRase with ONC-dimer should not have resulted in the conversion of ONC-dimer to ONC-monomer. In order to determine whether the purchased GSSGRase was contaminated with endogenous NADPH thus affording the reduction of ONC-dimer, the fluorescence emission spectrum of BIM GSSGRase was measured by excitation at 340nm (FIGURE 23). Solutions of known concentrations of FAD and NADPH were used as controls to determine the amount of each present in the enzyme preparation. The observed NADPH fluorescence was distinct from that of FAD in that the NADPH fluorescence was emitted at ~460nm whereas that for FAD at ~520nm. The enzyme was found to contain up to a ten-fold molar excess of NADPH with respect to GSSGRase. The purified rat liver enzyme was also found to contain NADPH (FIGURE 24). In both cases, the NADPH could not be removed by Sephadex G-25 chromatography and would appear to be a result of the purification procedure employed which utilizes 2', 5'-ADP Sepharose chromatography in which the enzyme is eluted by the application of NADPH to the column (Carlberg and Mannervik, 1985).

FIGURE 23

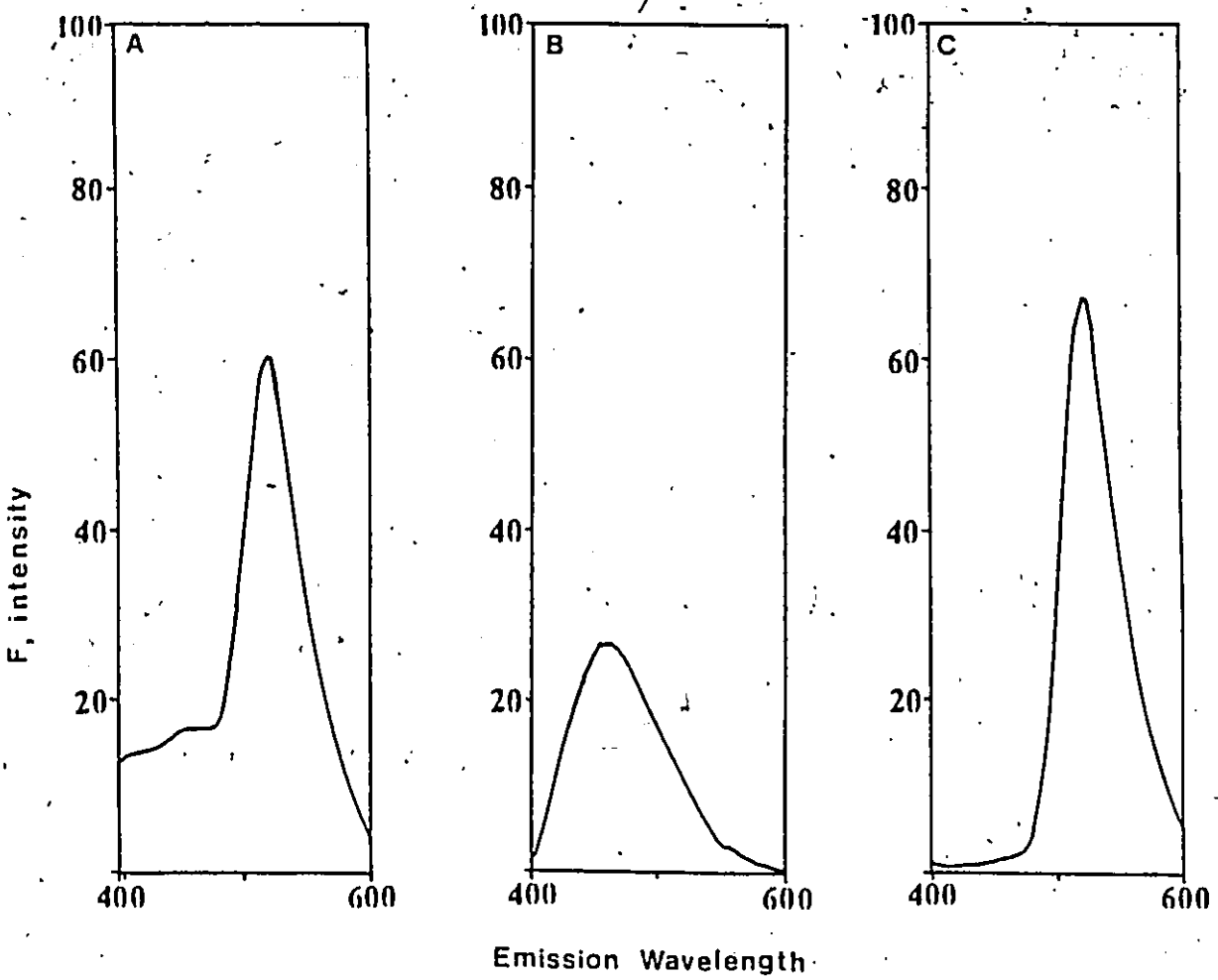


FIGURE 23: Fluorescence Emission Spectrum of Bovine Intestinal Mucosa Glutathione Reductase. Excitation wavelength used was 340nm. All spectra were performed in 100mM Tris-HCl, pH 7.0 at room temperature. Panel A: GSSGRase (5.2E-6M); Panel B: NADPH (7.4E-5M); Panel C: FAD (5.2E-6M).

FIGURE 24

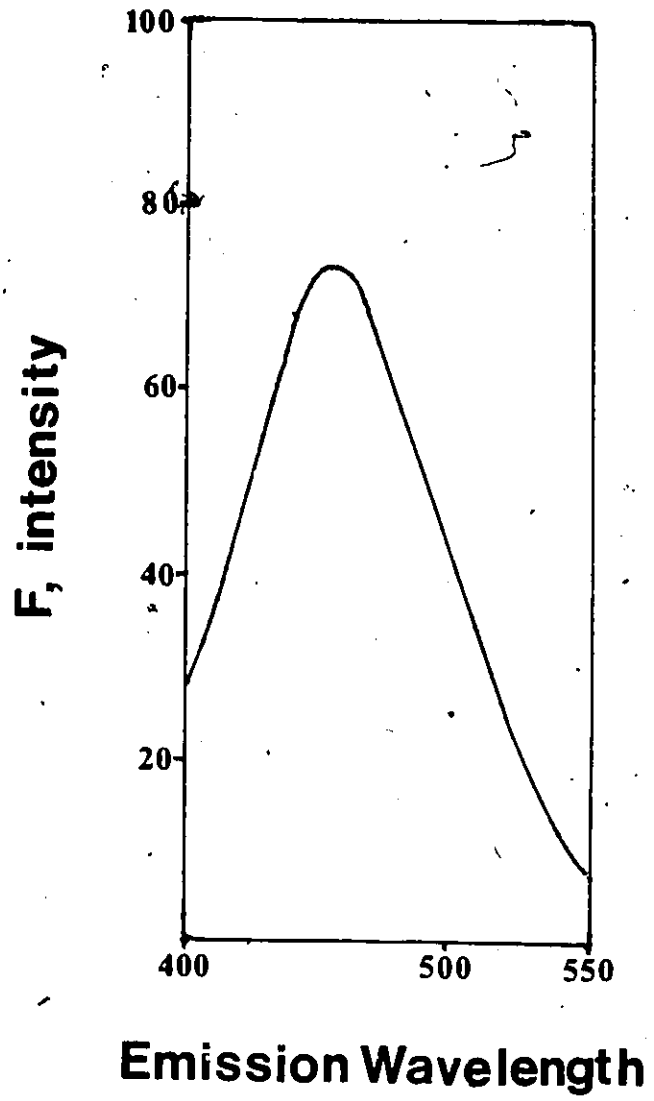


FIGURE 24: Fluorescence Emission Spectrum of Rat Liver Glutathione Reductase. Excitation wavelength used was 340nm. Spectrum was performed in 100mM Tris-HCl, pH 7.0 at room temperature. [GSSGRase] = 5.0E-7M.

7. Binding of Bovine Intestinal Mucosa Glutathione Reductase to Oncomodulin Sepharose

In order to determine whether the binding of glutathione reductase to oncomodulin was indeed calcium dependent, affinity chromatography was performed. The elution profile of glutathione reductase from oncomodulin monomer Sepharose is shown in FIGURE 25. Glutathione reductase (BIM) was applied to oncomodulin monomer Sepharose, preequilibrated with 100mM Tris-HCl, 1mM Ca^{2+} at pH 7.0. The enzyme was then eluted by the addition of EGTA. The eluted enzyme was still catalytically active after chromatography. When the enzyme was applied to the column in the presence of 1mM EGTA, no enzyme bound to the oncomodulin monomer Sepharose as all applied protein was accounted for in the wash fraction and no detectable protein (monitored at 280nm) or enzyme activity was observed upon the application of 1mM Ca^{2+} or 2M magnesium chloride.

8. Binding of Bovine Intestinal Mucosa Glutathione Reductase to Calmodulin Sepharose

In order to determine whether glutathione reductase interacted with calmodulin, calmodulin Sepharose affinity chromatography was used. In the presence of calcium

FIGURE 25

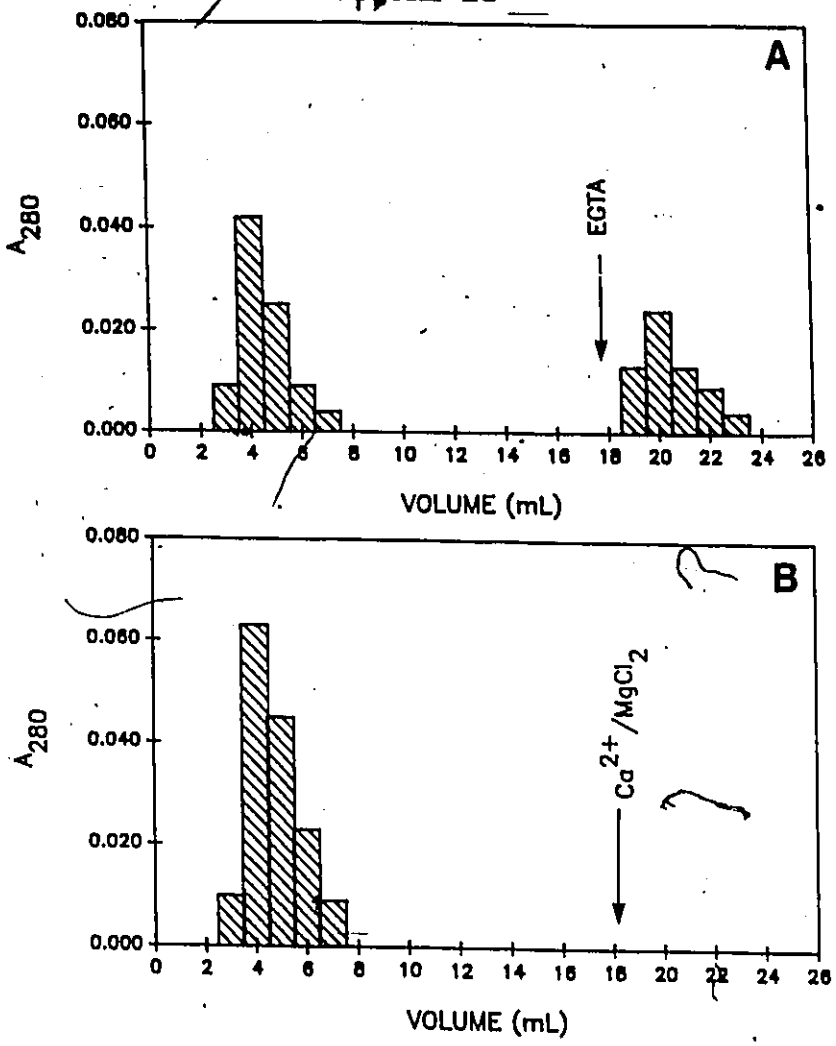


FIGURE 25: Elution Profile of Glutathione Reductase from Oncomodulin Sepharose. Protein was monitored at 280nm. Panel A: GSSGRase (200ug) in Tris-HCl (20mM), KCl (150mM) pH 7.5 containing 1.0mM Ca^{2+} was applied to oncomodulin Sepharose (preequilibrated in the same buffer). The enzyme (~140ug) was eluted by the application of the same buffer containing 2.0mM EGTA. Panel B: GSSGRase (200ug) (in Tris-HCl, 20mM; KCl, 150mM; EGTA, 1.0mM pH 7.5) was applied to oncomodulin Sepharose in the same buffer. Buffer containing 2mM Ca^{2+} or 2M magnesium chloride were then applied in an attempt to elute any bound protein.

essentially no glutathione reductase bound to the column as very little protein (monitored at 280nm) was detected upon the application of elution buffer (containing EGTA) (FIGURE 26). Essentially all of the applied protein was accounted for in the breakthrough fraction.

9. Bovine Intestinal Mucosa Glutathione Reductase Titrations

Bovine intestinal mucosa glutathione reductase (purchased from Sigma) was assayed in the presence of increasing amounts of reduced oncomodulin (FIGURE 27). ONC-monomer was found to inhibit the activity of glutathione reductase with 50% inactivation at ~1.3E-5M. The inhibition occurred only in the presence of Ca^{2+}; the addition of 1mM EDTA to the reaction mixture abolished the inhibition. Under identical conditions, calmodulin and rabbit parvalbumin were unable to alter glutathione reductase activity (FIGURE 28), indicating that this inhibition is an oncomodulin specific effect.

FIGURE 26

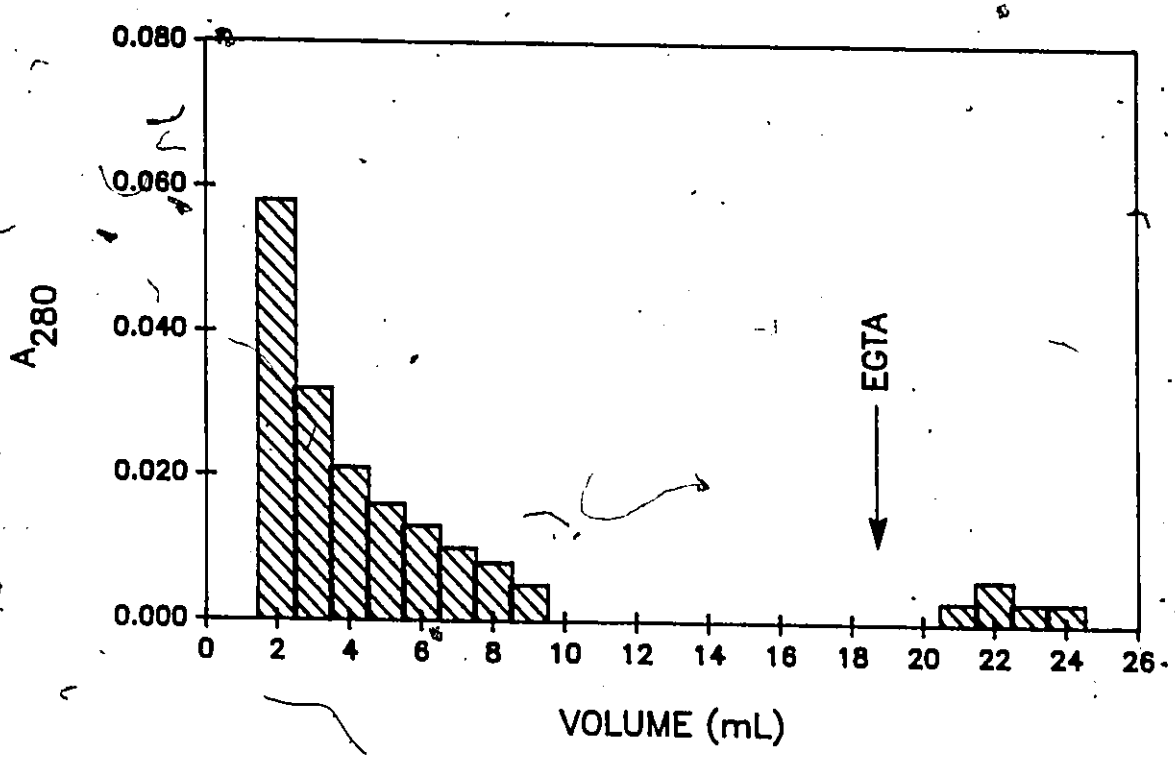


FIGURE 26: Elution Profile of Glutathione Reductase from Calmodulin Sepharose. Protein was monitored at 280nm. GSSGRase (300ug) in Tris-HCl (20mM), KCl (150mM) pH 7.5 containing 1.0mM Ca^{2+} was applied to calmodulin Sepharose (preequilibrated in the same buffer). Any bound protein was then eluted by the application of the same buffer containing 2.0mM EGTA.

FIGURE 27

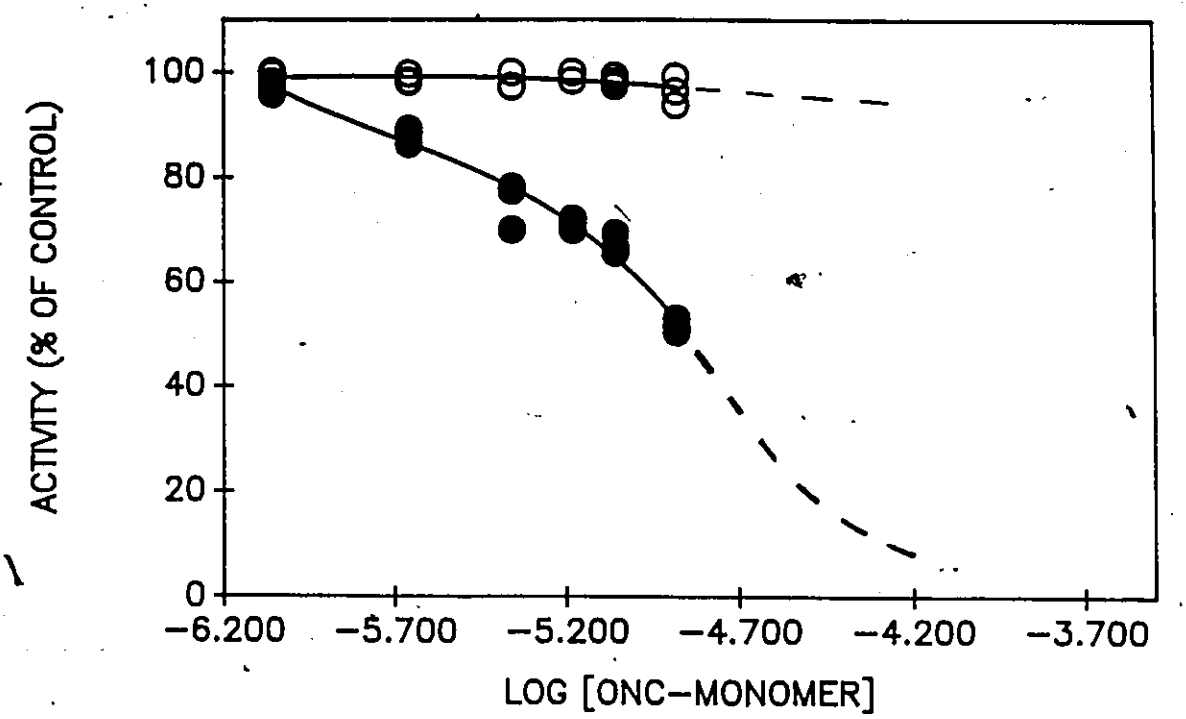


FIGURE 27: Titration of Bovine Intestinal Mucosa Glutathione Reductase Activity in the Presence of ONC-monomer in Calcium and EDTA. Increasing amounts of ONC-monomer were added to the reaction mixture containing GSSG (3E-4M), NADPH (1E-4M), GSSGRase (2E-9M) and 1.0mM Ca^{2+} (●) or 1mM EDTA (○). The reaction was allowed to proceed for 1 minute at 30°C and stopped by the addition of 50uL of 10% SDS. The amount of NADPH consumed was determined at 340nm against a blank containing no GSSG. Values are given as a percentage of that obtained in the absence of added protein.

FIGURE 28

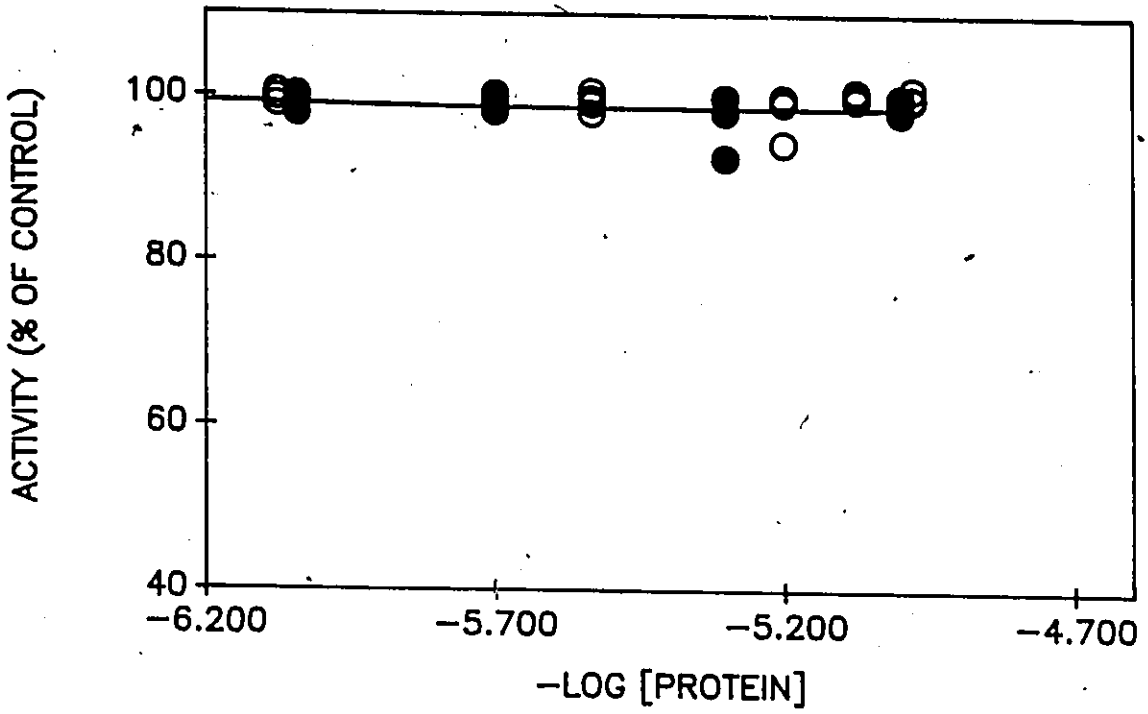


FIGURE 28: Titration of Bovine Intestinal Mucosa Glutathione Reductase Activity in the Presence of Calmodulin and Rabbit Parvalbumin. Increasing amounts of calmodulin (●) and rabbit parvalbumin (○) were added to the reaction mixture containing GSSG (3E-4M), NADPH (1E-4M), GSSGRase (2E-9M) and 1.0mM Ca^{2+}. The reaction was allowed to proceed for 1 minute at 30°C and stopped by the addition of 50uL of 10% SDS. The amount of NADPH consumed was determined at 340nm against a blank containing no GSSG. Values are given as a percentage of that obtained in the absence of added protein.

10. Kinetics of Bovine Intestinal Mucosa Glutathione Reductase

The published assay which is used for glutathione reductase activity employs 2mM EDTA (Carlberg and Mannervik, 1985). Since the proteins which are to be tested are Ca^{2+} binding proteins, the effect of calcium on glutathione reductase was determined (FIGURE 29). The apparent K_m in the presence of calcium was essentially the same (9.03E-5 +/- 0.07E-5M) as that obtained in the presence of EDTA (8.59E-5 +/- 1.4E-5M). The apparent V_{max} obtained in the presence of calcium was markedly lower (0.03037 +/- 2.2E-5mol/min/mg) compared to EDTA (0.05075 +/- 0.0005mol/min/mg). The apparent K_m obtained in the presence of EDTA was essentially the same as that reported for the calf liver enzyme (10E-5M) (Carlberg and Mannervik, 1981). The addition of 1E-6M calmodulin to the reaction mixture had no effect on the utilization of GSSG by glutathione reductase ($K_{m,app}$ = 9.41E-5 +/- 0.05E-5M: $V_{max,app}$ = 0.03106 +/- 1.1E-4mol/min/mg) (FIGURE 30).

The effect of the addition of 1E-6M ONC-monomer to the GSSGRase assay is shown in FIGURE 31. The apparent K_m for GSSG in the presence of reduced oncomodulin (8.89 +/- 0.15E-5M) was unchanged from that obtained in the presence of calcium alone. However, the apparent V_{max} obtained was significantly lower (0.01905 +/- 0.0020mol/min/mg). The

FIGURE 29

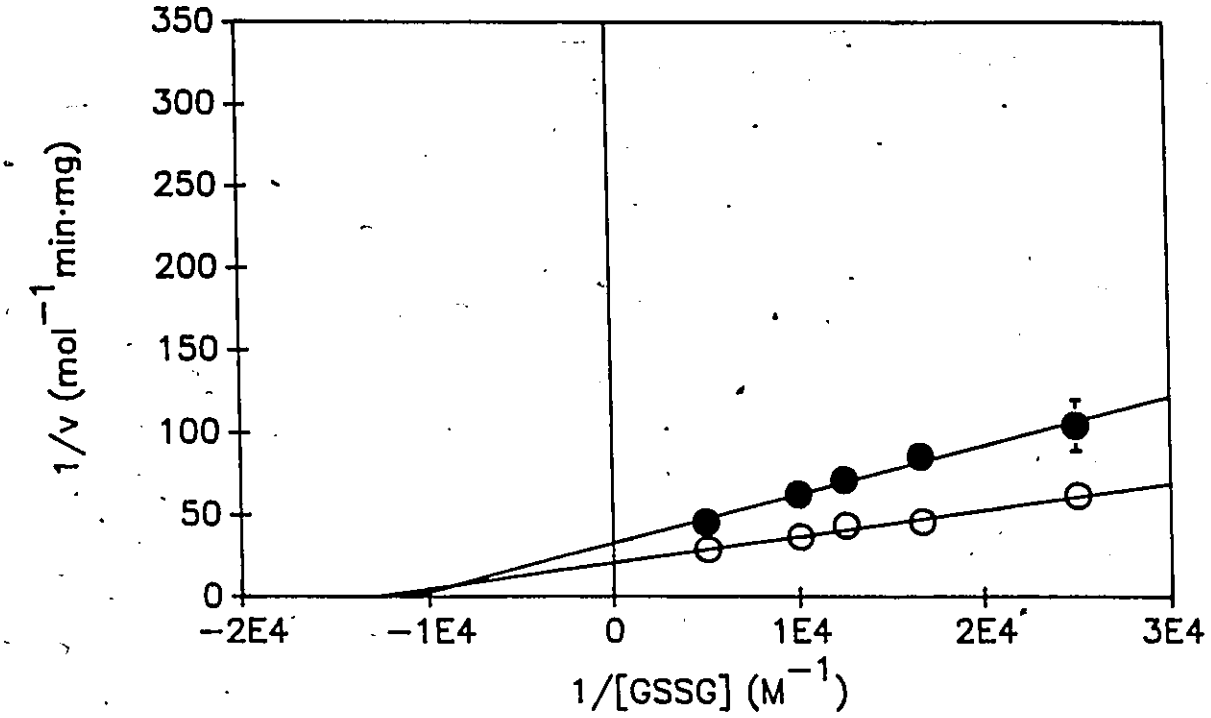


FIGURE 29: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in Calcium and EDTA. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH and 2E-9M GSSGRase. 1.0mM EDTA: ○ ; 1.0mM Ca^{2+}: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 30

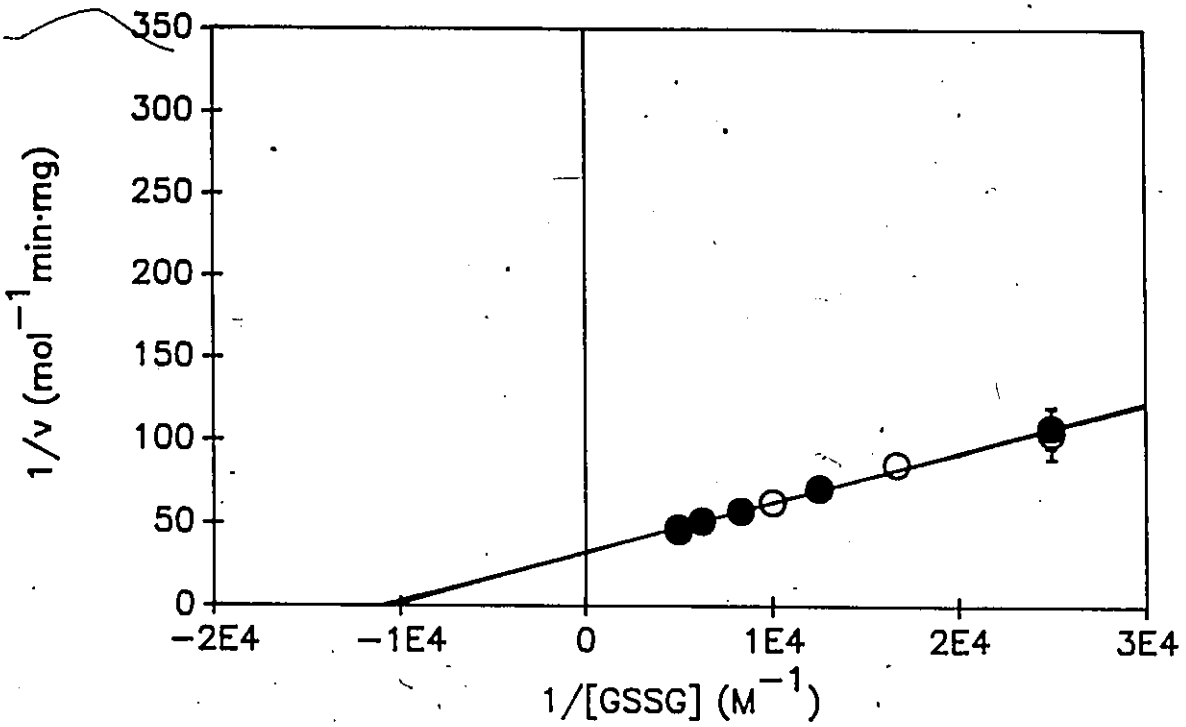


FIGURE 30: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in the Presence of Calmodulin. Kinetics were performed at 30 °C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M calmodulin and 2E-9M GSSGRase. 1.0mM Ca^{2+}: ○; CaM: ●. The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 31

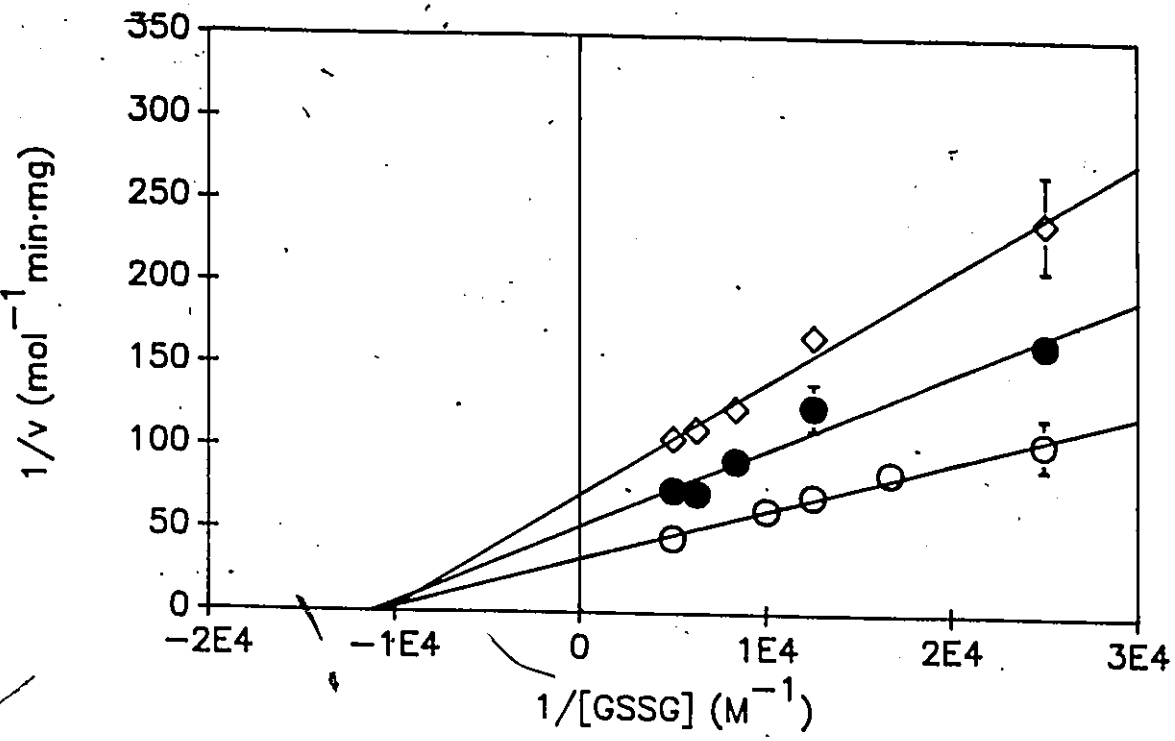


FIGURE 31: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in the Presence of ONC-monomer. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+} and 6E-9M GSSGRase. Ca^{2+}: ○; 1E-6M ONC-monomer: ●; 1E-5M ONC-monomer: ◇. The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

inhibition was dose dependent as the inclusion of 1E-5M ONC-monomer (FIGURE 31) resulted in a further decrease in the apparent V_{max} (0.01395 +/- 0.0016mol/min/mg) while leaving the apparent K_m unchanged (9.51 +/- 0.15E-5M). This suggests that ONC-monomer is a noncompetitive inhibitor of GSSG utilization by BIM GSSGRase. The apparent K_i for reduced oncomodulin was calculated to be 1.64E-6M. The inclusion of 1E-6M S-carboxymido-ONC, where the cys-18 thiol of oncomodulin has been chemically blocked by iodoacetamide, in the assay resulted in kinetics which were superimposable on those obtained in the presence of 1E-6M ONC-monomer ($K_{m,app}$ = 9.07 +/- 0.16E-5M: $V_{max,app}$ = 0.01829 +/- 0.00024mol/min/mg) (FIGURE 32).

Carp skeletal muscle beta parvalbumin (cPV) (pI = 4.25) has 52 amino acids in identical positions to rat oncomodulin including the cys-18 thiol (Kretsinger, 1980). cPV has the ability to dimerize via this thiol in the presence of EGTA. The kinetics obtained in the presence of 1E-6M oxidized cPV were the same as those in the presence of Ca^{2+} alone ($K_{m,app}$ = 9.12E-5 +/- 0.12E-5M: $V_{max,app}$ = 0.03083 +/- 0.0002mol/min/mg) (FIGURE 33). Rabbit skeletal muscle parvalbumin is also highly homologous to rat oncomodulin, having 50 amino acids in identical positions but lacking the cys-18 thiol which is replaced by phenylalanine (Capony et al., 1976). When 1E-6M rabbit parvalbumin was added to the assay mixture, no effect was observed ($K_{m,app}$ =9.89E-5 +/-

FIGURE 32

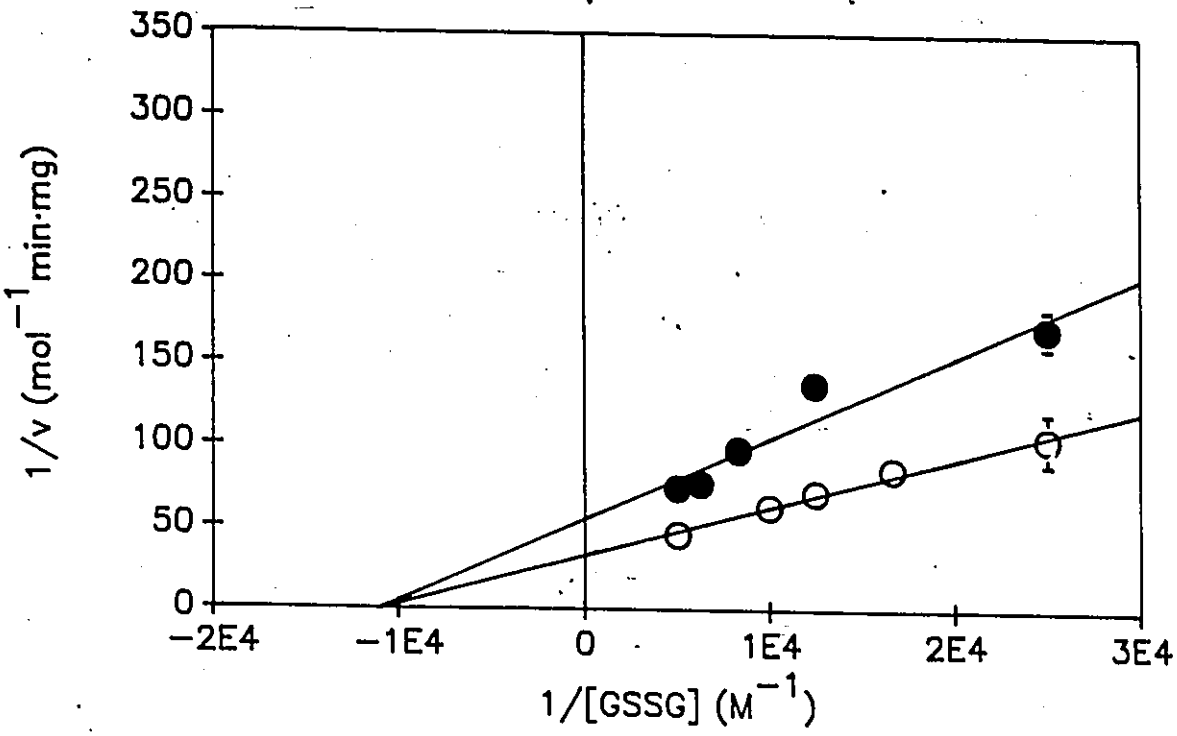


FIGURE 32: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in the Presence of S-carboxymido-DNC. Kinetics were performed at 30 °C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M S-carboxymido-DNC and 6E-9M GSSGRase. Ca^{2+}: ○; S-carboxymido-DNC: ● The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 33

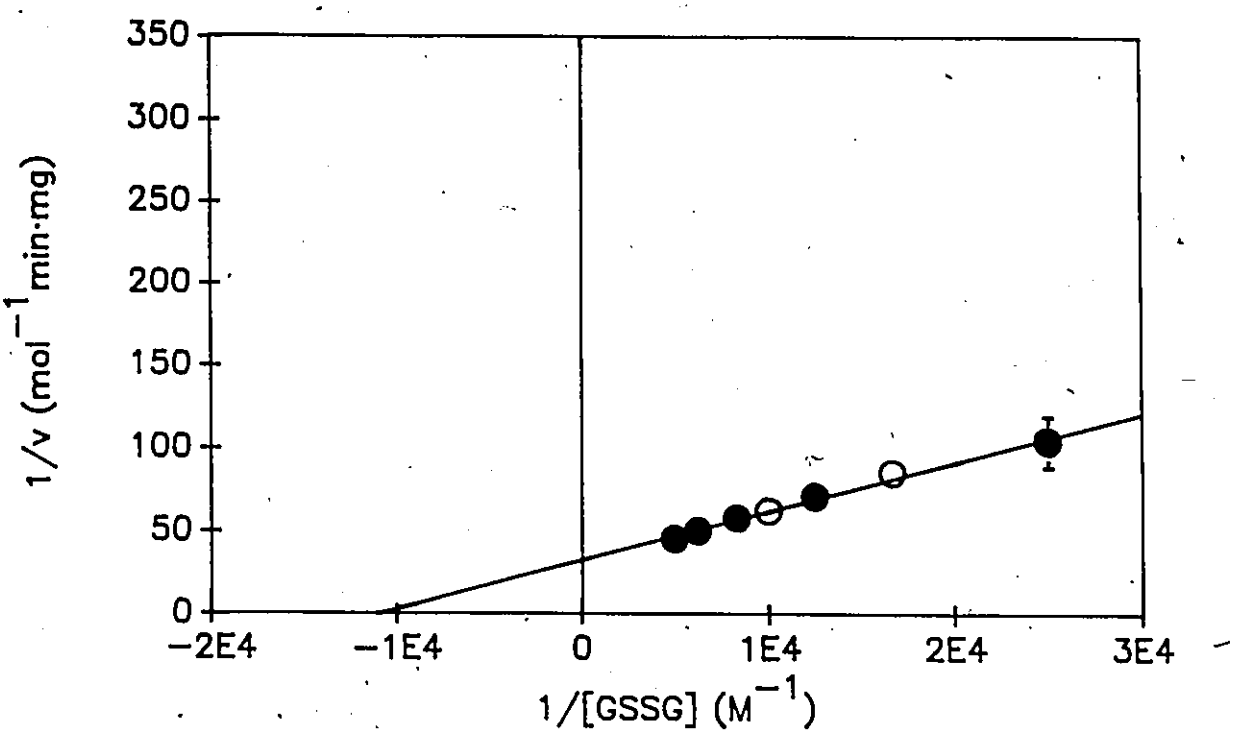


FIGURE 33: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in the Presence of Carp Parvalbumin Dimer. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M cPV-dimer and 2E-9M GSSGRase. Ca^{2+}: ○ ;cPV-dimer: ●. The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

1.01E-5M: $V_{max.app}$ = 0.03163 +/- 0.0019mol/min/mg) (FIGURE 34). Therefore the site of interaction of oncomodulin with GSSGRase is lacking in the rabbit and carp parvalbumins. The lacking cys-18 thiol in rabbit parvalbumin was not essential since SAM-ONC was able to inhibit GSSGRase to the same extent as reduced oncomodulin.

A summary of the kinetic parameters obtained for the bovine intestinal mucosa enzyme are given in Table 1.

11. Effect of Oncomodulin Monomer on NADPH Fluorescence

In order to ensure that the inhibition of GSSGRase by oncomodulin monomer was not a result of nonspecific sequestering of NADPH by oncomodulin, the fluorescence of NADPH in the presence and absence of oncomodulin was determined (FIGURE 35). The fluorescence of NADPH (1E-4M) was determined in the presence and absence of 5E-5M oncomodulin and rabbit parvalbumin (excitation wavelength = 340nm). Incubation with either protein in the presence of calcium resulted in only a 5% increase in the fluorescence emission intensity with no apparent change in emission wavelength maximum. Since an identical spectrum was obtained in the presence of rabbit parvalbumin which has no effect on GSSGRase activity, the slight alteration of the fluorescence emission spectrum is not indicative of an

FIGURE 34

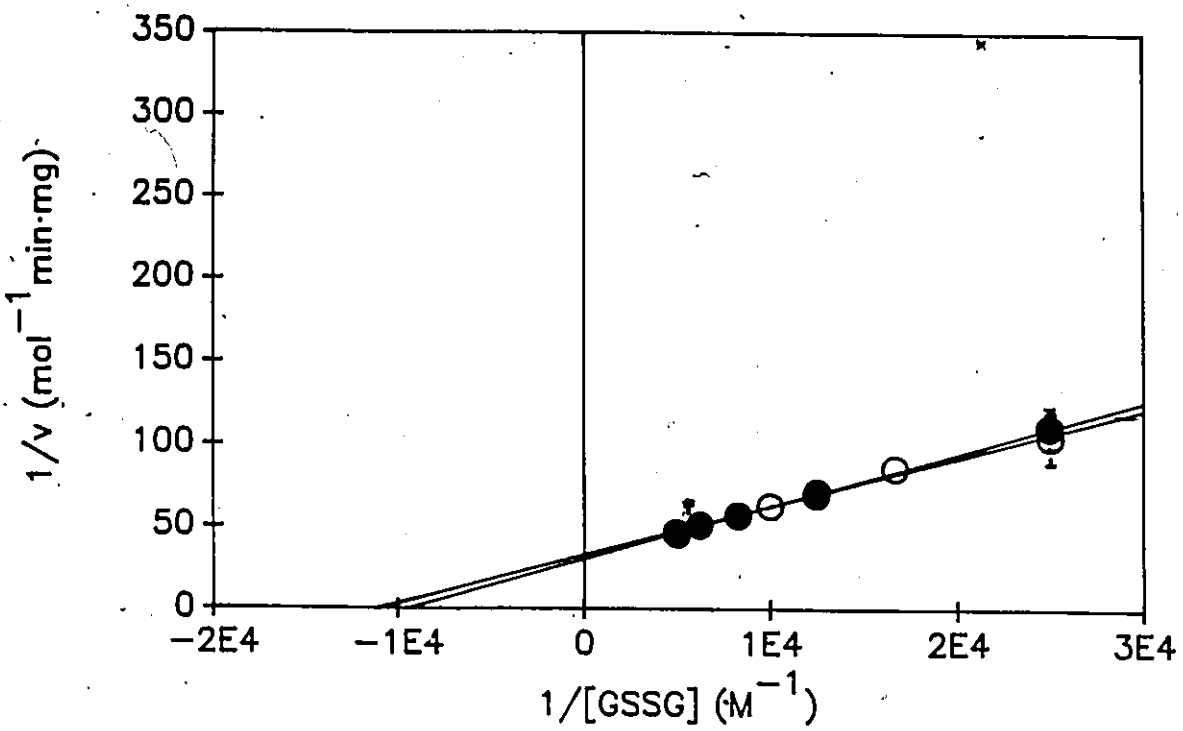


FIGURE 34: Kinetics of Bovine Intestinal Mucosa Glutathione Reductase in the Presence of Rabbit Parvalbumin. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M rPV and 2E-9M GSSGRase. Ca^{2+}: ○ ;rPV: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

TABLE 1

KINETIC PARAMETERS OF BOVINE INTESTINAL MUCOSA GLUTATHIONE REDUCTASE UTILIZATION OF GSSG

	$K_{m,app}$ (M)	$V_{max,app}$ (mol/min/mg)
EDTA	8.59 +/- 1.4E-5	0.05075 +/- 0.0005
Ca^{2+}	9.03 +/- 0.07E-5	0.03037 +/- 0.00022
CaM (1E-6M)	9.41 +/- 0.05E-5	0.03106 +/- 0.00011
ONC-monomer (1E-6M)	8.89 +/- 0.15E-5	0.01905 +/- 0.0020
ONC-monomer (1E-5M)	9.51 +/- 0.15E-5	0.01395 +/- 0.0016
S-carboxymido-ONC (1E-6M)	9.07 +/- 0.16E-5	0.01829 +/- 0.0024
rPV (1E-6M)	9.89 +/- 1.01E-5	0.03163 +/- 0.0019
cPV-dimer (1E-6M)	9.12 +/- 0.12E-5	0.03083 +/- 0.0002

Kinetic parameters obtained are the average of the values obtained for runs of quadruplicate samples. Standard deviation is calculated from the values obtained between runs.

FIGURE 35

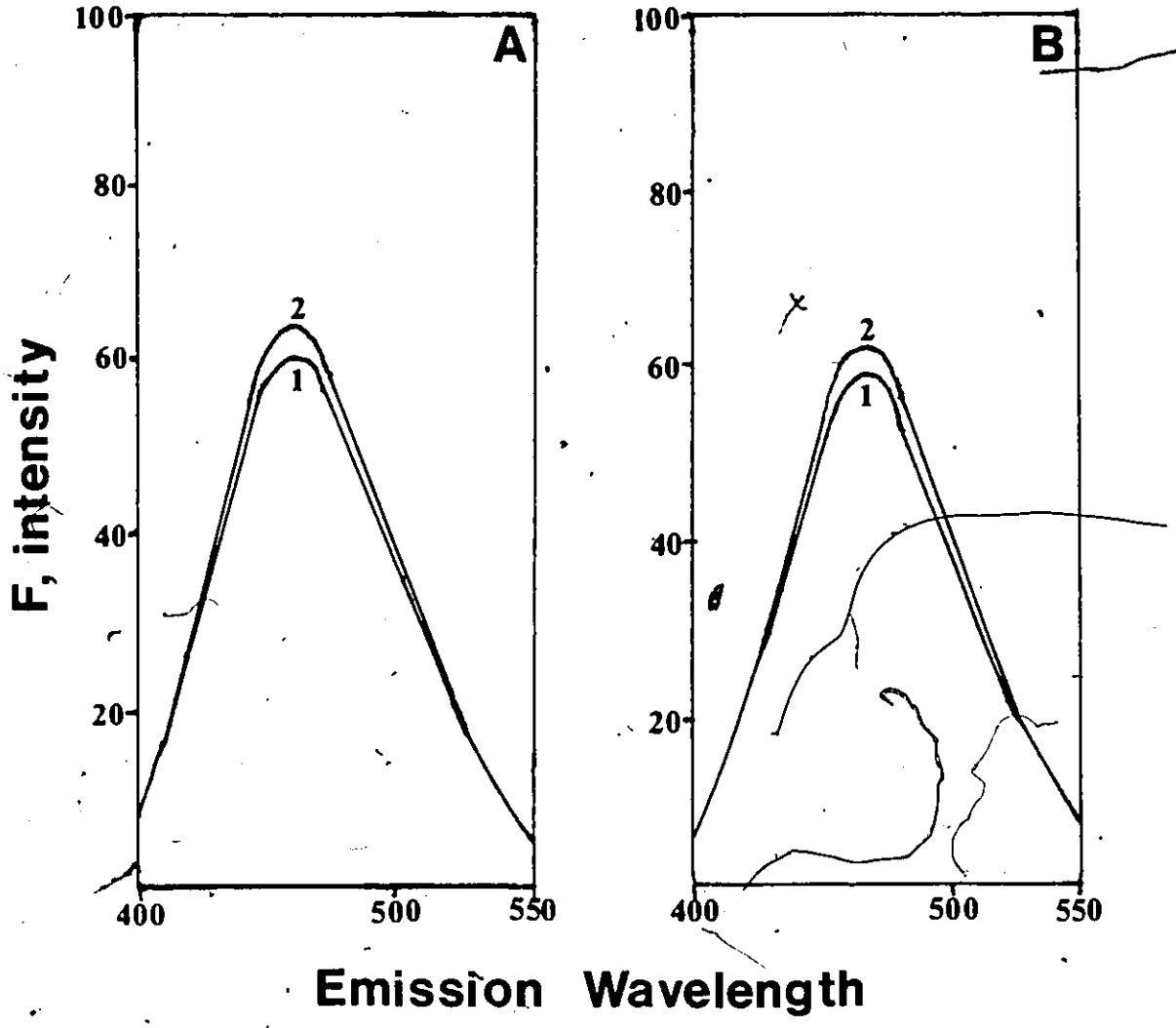


FIGURE 35: Effect of Oncomodulin and Rabbit Parvalbumin on NADPH Fluorescence. Excitation wavelength used was 340nm. All spectra were performed in 100mM Tris-HCl, pH 7.0 containing 1mM Ca^{2+} at room temperature. Panel A: 1: 5E-5M NADPH, 2: 5E-5M NADPH plus 5E-5M ONC-monomer; Panel B: 1: 5E-5M NADPH, 2: 5E-5M NADPH plus 5E-5M rPV.

interaction which interferes with NADPH utilization.

12. Purification of Rat Liver Glutathione Reductase

The purification of glutathione reductase from rat liver is summarized in Table 2. A typical purification from 25-10g rat livers resulted in ~0.5mg of glutathione reductase. A 10% SDS-PAGE of the purification steps is also illustrated (FIGURE 36).

13. Rat Liver Glutathione Reductase Titrations

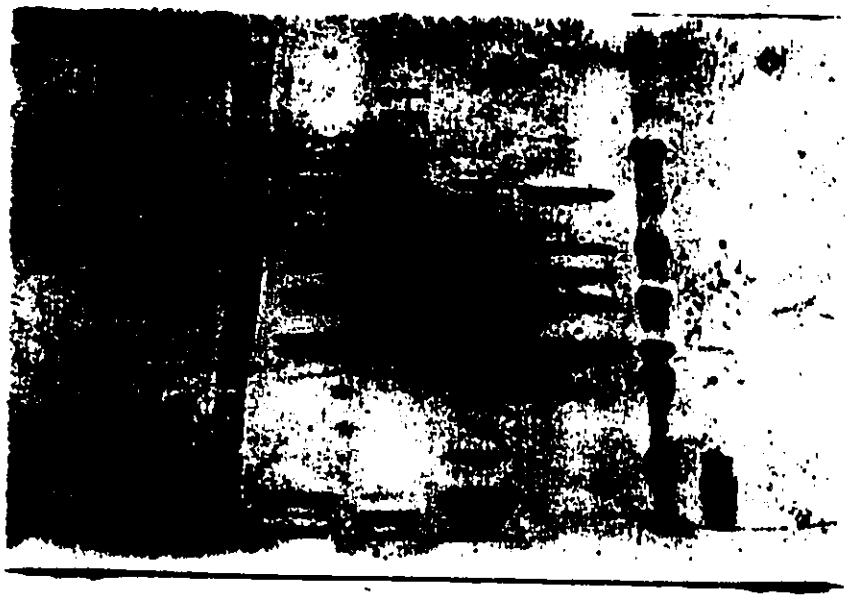
The effect of increasing amounts of reduced oncomodulin on rat liver GSSGRase is shown in FIGURE 37. ONC-monomer was found to inhibit the enzyme with 50% inactivation occurring at ~1E-5M. When the assay was performed in the presence of 1mM EDTA, no inactivation was observed indicating that the inhibition is calcium dependent. The inclusion of increasing amounts of calmodulin and rabbit parvalbumin in the assay mixture had no effect on GSSGRase activity (FIGURE 38).

TABLE 2

PURIFICATION OF RAT LIVER GLUTATHIONE REDUCTASE

	Volume (mL)	Total Protein (mg)	Activity (mol/min/mg)	Fold Purification
Supernatant	2000	10200	3.68E-5	--
CM-cellulose	500	105	1.18E-4	97.1
G75 Sephadex.	125	71.2	2.12E-4	143.3
ADP-Sepharose	4	0.52	2.86E-2	1.96E4

FIGURE 36



1 2 3 4 5 6 7 8

FIGURE 36: 10% SDS-PAGE of Purification Steps of Rat Liver Glutathione Reductase. Lane 1,8: molecular weight standards: Bovine serum albumin (66200), ovalbumin (42700), glyceraldehyde 3-phosphate dehydrogenase (35000), carbonic anydrase (28900), trypsinogen (24000), soybean trypsin inhibitor (20100) and lactalbumin (14200); Lane 2: supernatent after centrifugation; Lane 3: CM-cellulose breakthrough fraction; Lane 4: CM-cellulose peak; Lane 5: G-75 Sephadex peak 1 (active); Lane 6: G-75 Sephadex peak 2 (no GSSGRase activity); Lane 7: post ADP Sepharose (final preparation). All GSSGRase samples contained ~30ug of protein.

FIGURE 37

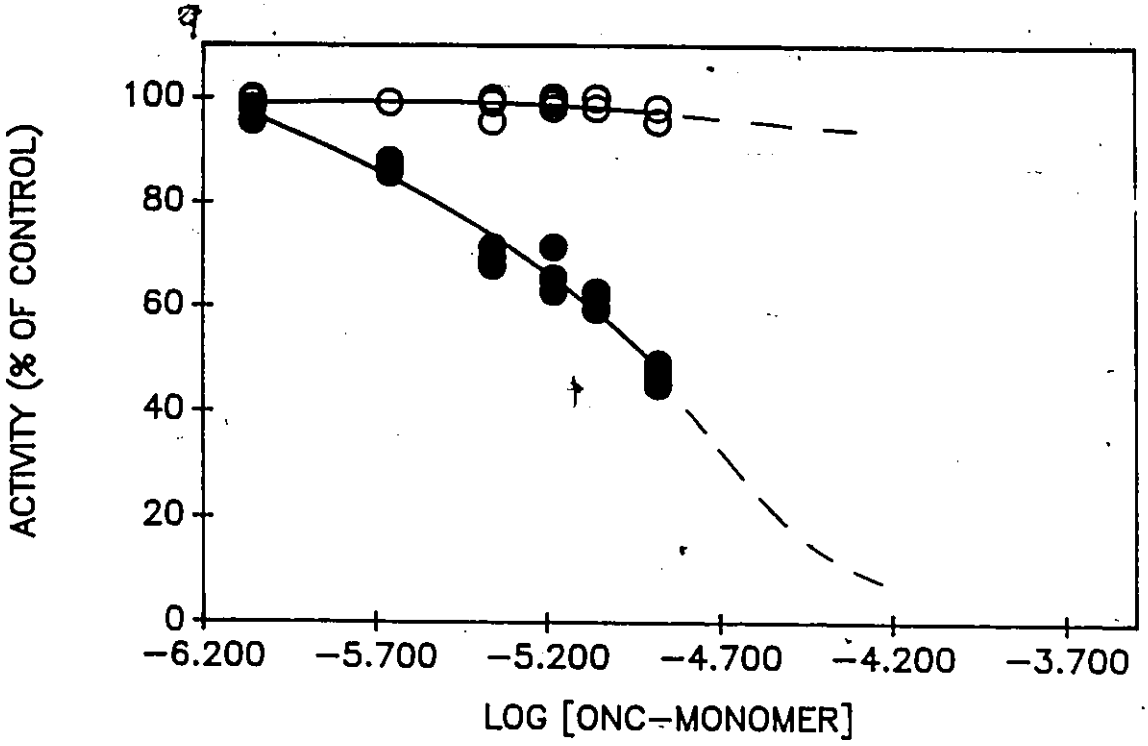


FIGURE 37: Titration of Rat Liver Glutathione Reductase Activity in the Presence of ONC-monomer in Calcium and EDTA. Increasing amounts of ONC-monomer were added to the reaction mixture containing GSSG (3E-4M), NADPH (1E-4M), GSSGRase (4.6E-9M) and 1.0mM Ca^{2+} (●) or 1mM EDTA (○). The reaction was allowed to proceed for 1 minute at 30°C and stopped by the addition of 50uL of 10% SDS. The amount of NADPH consumed was determined at 340nm against a blank containing no GSSG. Values are given as a percentage of that obtained in the absence of added protein.

FIGURE 38

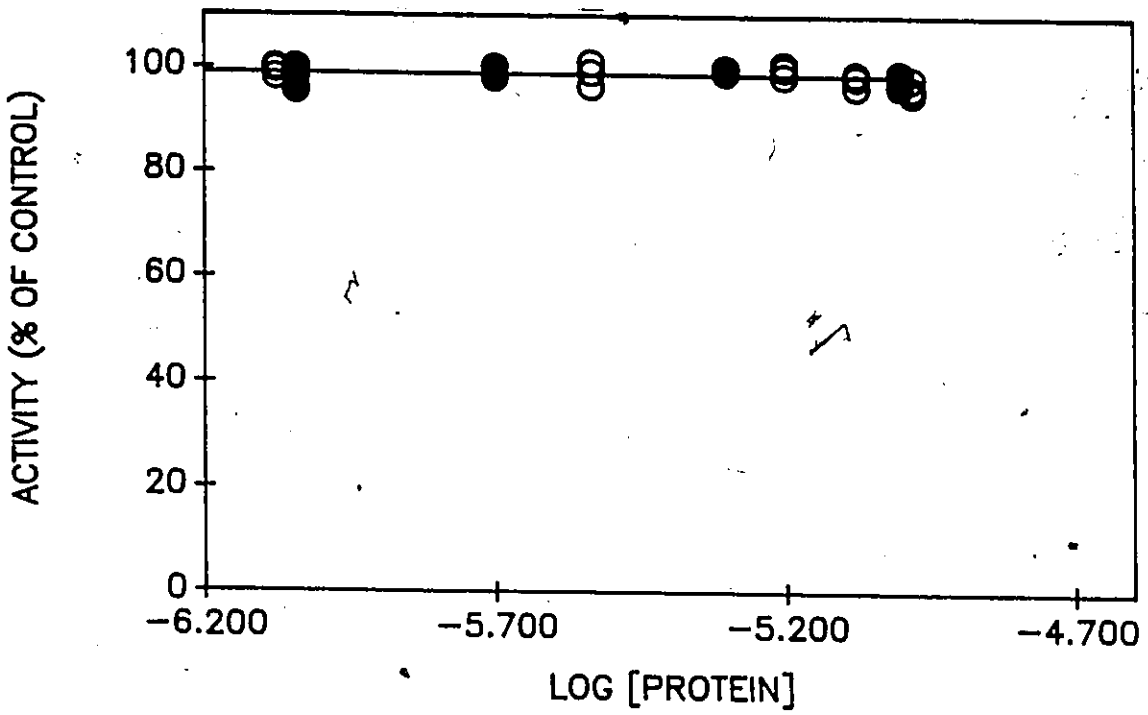


FIGURE 38: Titration of Rat Liver Glutathione Reductase Activity in the Presence of Calmodulin and Rabbit Parvalbumin. Increasing amounts of calmodulin (●) and rabbit parvalbumin (○) were added to the reaction mixture containing GSSG (3E-4M), NADPH (1E-4M), GSSGRase (4.6E-9M) and 1.0mM Ca^{2+}. The reaction was allowed to proceed for 1 minute at 30° C and stopped by the addition of 50uL of 10% SDS. The amount of NADPH consumed was determined at 340nm against a blank containing no GSSG. Values are given as a percentage of that obtained in the absence of added protein.

14. Rat Liver Glutathione Reductase Kinetics

The results obtained with purified rat liver GSSGRase were essentially the same as those obtained with the purchased BIM enzyme. The inclusion of Ca^{2+} in the assay resulted in an apparent K_m of 6.71E-5 +/- 0.40E-5M which was essentially the same as that obtained in EDTA ($K_{m,app}$ = 5.01E-5 +/- 0.29E-5M) (FIGURE 39). The apparent K_m in the presence of EDTA agreed well with published values (K_m = 2.63E-5M: Carlberg et al., 1981). The apparent V_{max} was decreased from 0.02345 +/- 0.0004mol/min/mg in EDTA to 0.01014 +/- 0.0003mol/min/mg in Ca^{2+}. As with the BIM GSSGRase, the kinetics obtained in the presence of calmodulin were superimposable on those obtained in the presence of calcium alone ($K_{m,app}$ = 6.8E-5 +/- 0.23E-5M: $V_{max,app}$ = 0.01347 +/- 0.0004mol/min/mg) (FIGURE 40).

The inclusion of 1E-6M oncomodulin monomer in the assay resulted in no change in the apparent K_m (6.21E-5 +/- 0.59E-5M) whereas the apparent V_{max} was decreased (0.00716 +/- 0.0004mol/min/mg) indicating that ONC-monomer is a noncompetitive inhibitor of RL GSSGRase as well as BIM GSSGRase (FIGURE 41). The inhibition was again dose dependent as the inclusion of 1E-5M ONC-monomer resulted in a further decrease in the apparent V_{max} (0.00551 +/- 0.0004mol/min/mg) with no change in the apparent K_m (6.09E-5 +/- 0.55E-5M) (FIGURE 41). The apparent K_i for reduced

FIGURE 39

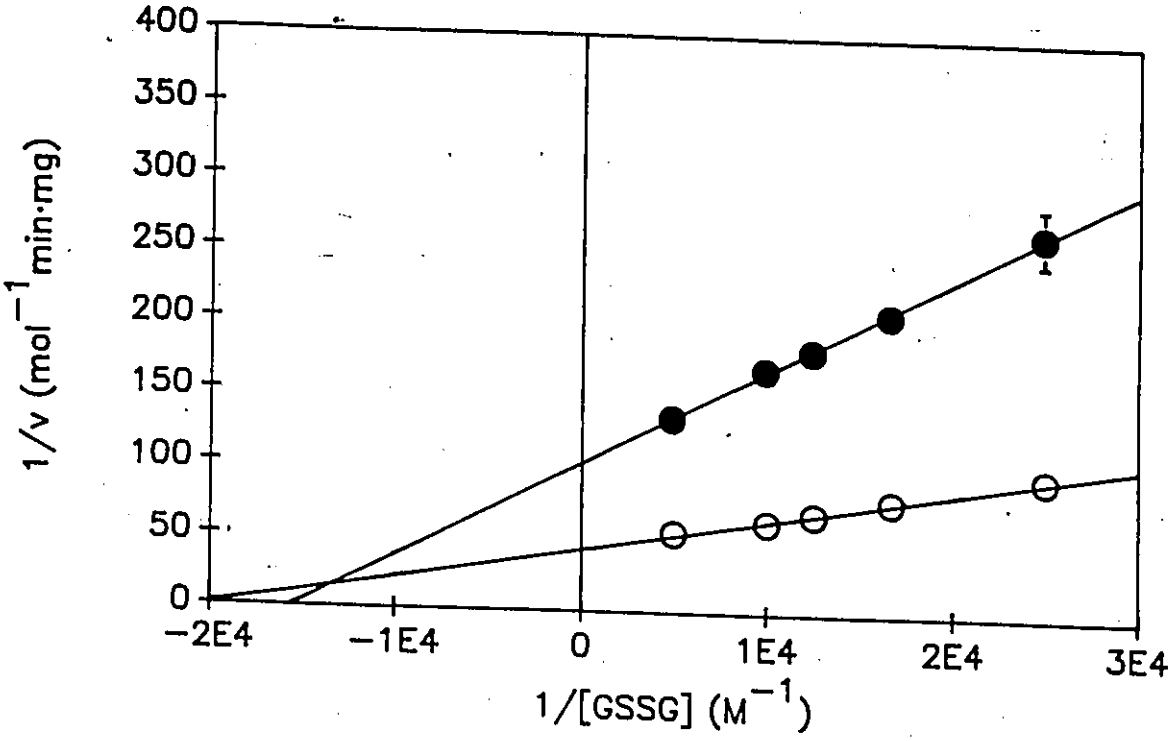


FIGURE 39: Kinetics of Rat Liver Glutathione Reductase in the Presence of Calcium and EDTA. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, and 4.6E-9M GSSGRase. 1.0mM EDTA: ○ ; 1.0mM Ca^{2+}: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 40

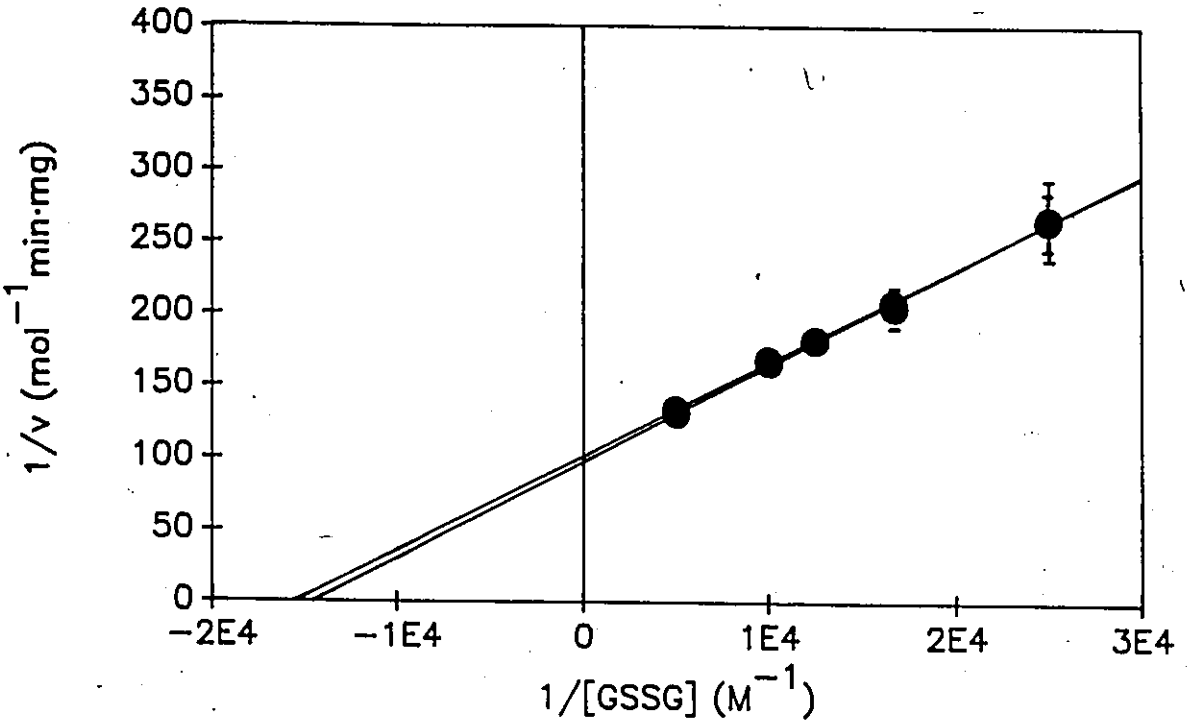


FIGURE 40: Kinetics of Rat Liver Glutathione Reductase in the Presence of Calmodulin. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1E-6M calmodulin and 4.6E-9M GSSGRase. 1.0mM Ca^{2+}: ○ ; CaM: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 41

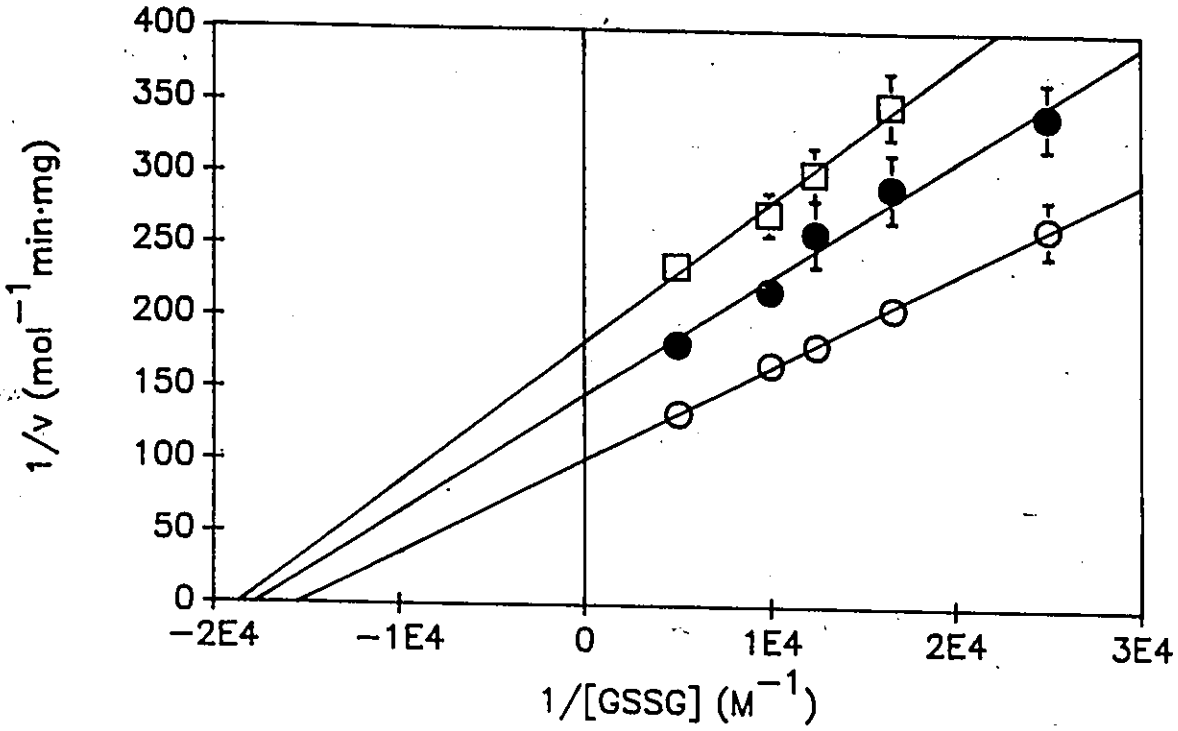


FIGURE 41: Kinetics of Rat Liver Glutathione Reductase in the Presence of ONC-monomer in Calcium. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+} and 4.6E-9M GSSGRase. Ca^{2+}; ○ ; 1E-6M ONC-monomer; ● ; 1E-5M ONC-monomer; □ . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

oncomodulin was calculated to be 2.4E-6M. Kinetics performed with 1E-6M ONC-monomer in EDTA were superimposable on those of EDTA alone further illustrating the interaction is Ca^{2+} dependent (FIGURE 42). The inclusion of 1E-6M S-carboxymido-ONC in the assay resulted in inhibition of the enzyme in the same manner as ONC-monomer ($K_{m,app}$ = 5.23E-5M +/- 0.17E-5M: $V_{max,app}$ = 0.00685 +/- 0.0008mol/min/mg) (FIGURE 43). As with BTM GSSGRase, the free thiol at position 18 of oncomodulin does not appear to be involved in the inhibition since chemical modification of this amino acid did not alter the kinetic parameters obtained.

Rabbit parvalbumin and oxidized carp skeletal muscle beta parvalbumin were also tested for the ability to inhibit rat liver GSSGRase. Both were found to have no effect on the enzyme again illustrating that the effect is oncomodulin specific and is not shared by the closely related beta parvalbumins (FIGURE 44 and 45).

A summary of the kinetic parameters obtained is given in Table 3.

15. Summary of Results

Oncomodulin contains a single thiol at position 18 of its primary structure. The reactivity of the thiol was found to be dependent on the conformation of the protein as

FIGURE 42

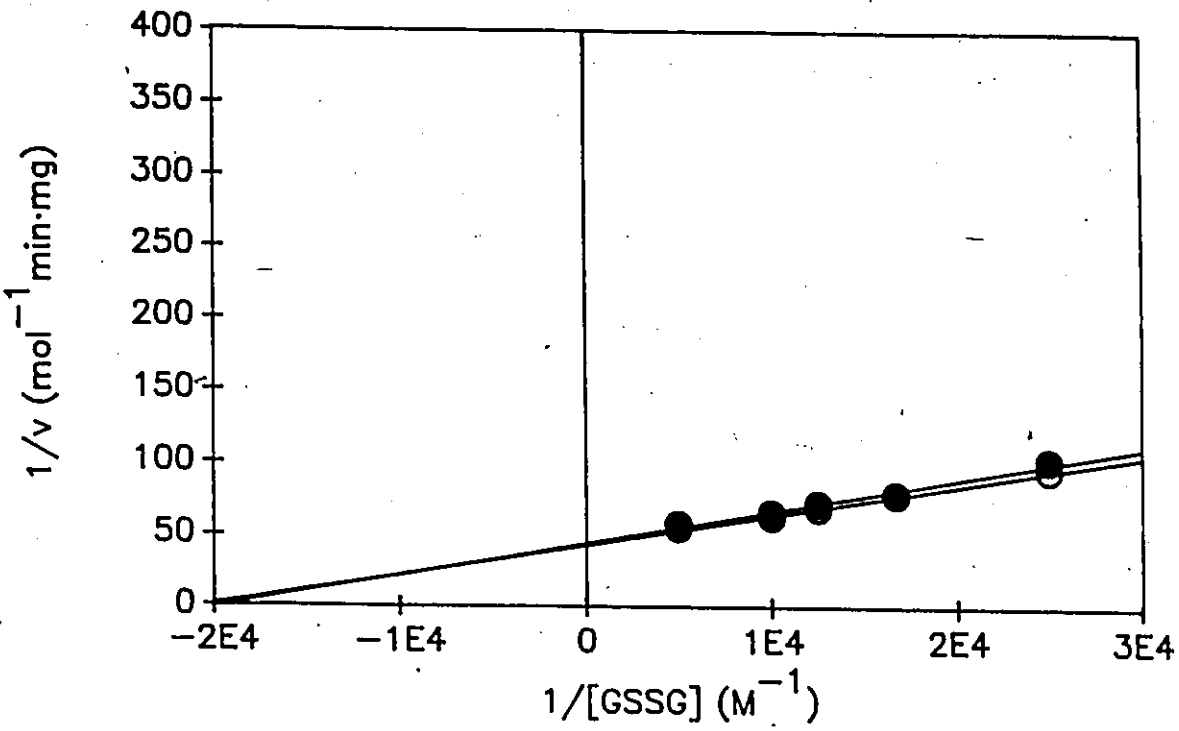


FIGURE 42: Kinetics of Rat Liver Glutathione Reductase in the Presence of ONC-monomer in EDTA. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM EDTA, 1E-6M ONC-monomer and 4.6E-9M GSSGRase. EDTA: ○ ; ONC-monomer: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 43

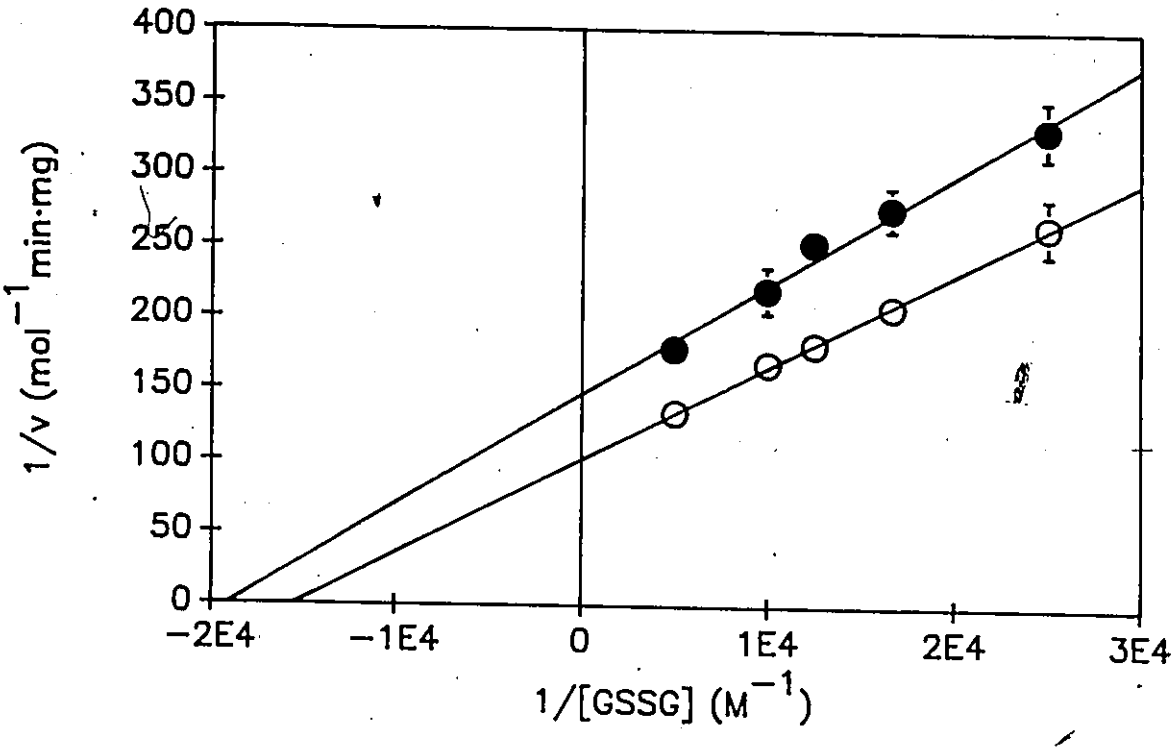


FIGURE 43: Kinetics of Rat Liver Glutathione Reductase in the Presence of S-carboxymido-ONC. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M S-carboxymido-ONC and 4.6E-9M GSSGRase. Ca^{2+}: ○ ; S-carboxymido-ONC: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 44

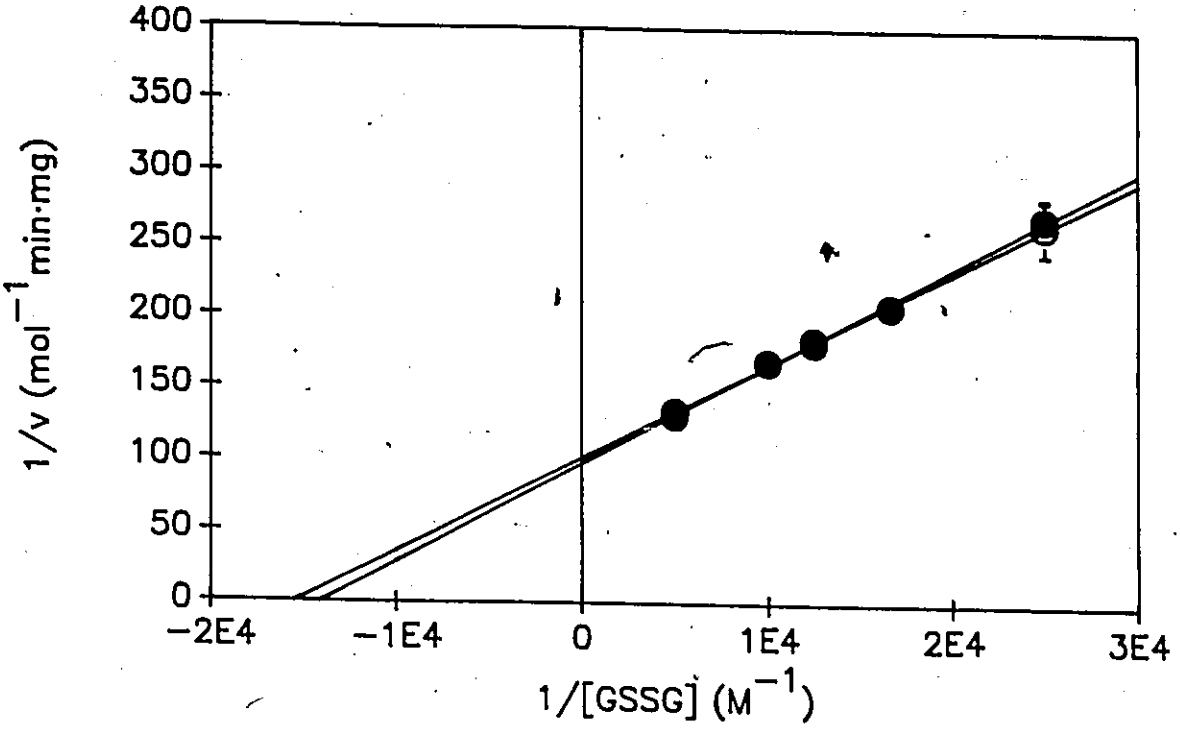


FIGURE 44: Kinetics of Rat Liver Glutathione Reductase in the Presence of Rabbit Parvalbumin. Kinetics were performed at 30°C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M rPV and 4.6E-9M GSSGRase. Ca^{2+}: ○ ; 1E-6M rPV: ● . The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

FIGURE 45

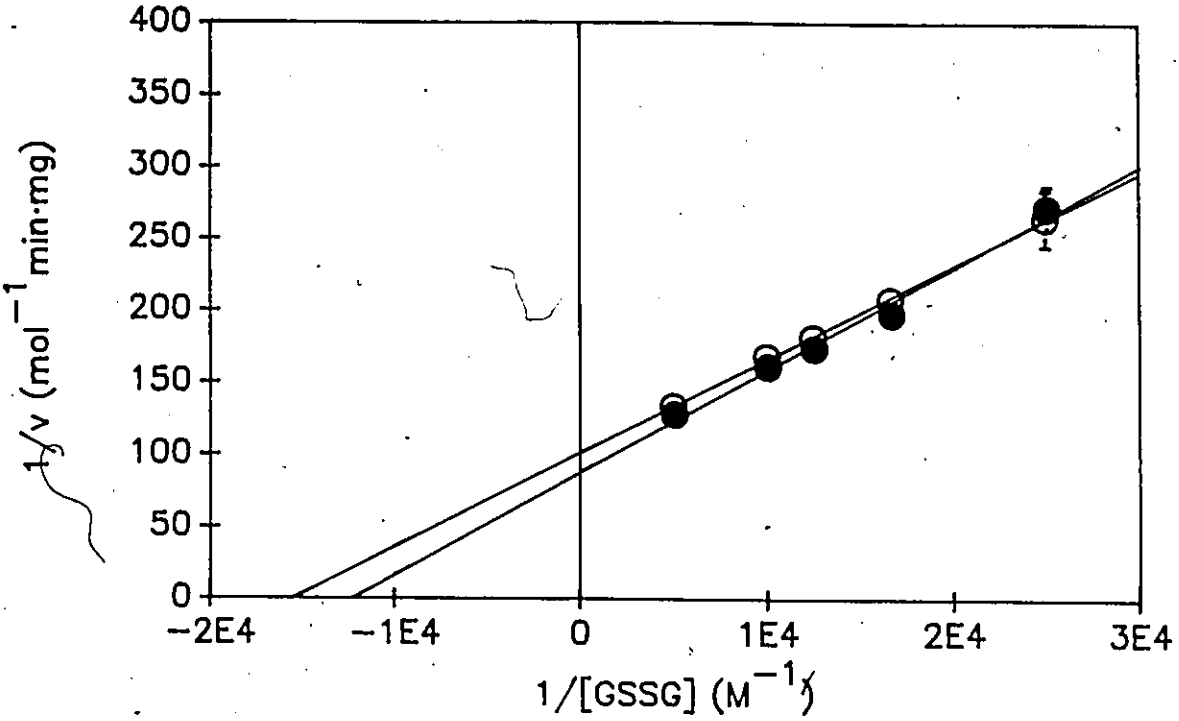


FIGURE 45: Kinetics of Rat Liver Glutathione Reductase in the Presence of Carp Parvalbumin Dimer. Kinetics were performed at 30 °C for 1 minute incubations which were stopped by the addition of SDS (0.5% final). The assay mixture contained 1E-4M NADPH, 1.0mM Ca^{2+}, 1E-6M cPV-dimer and 4.6E-9M GSSGRase. Ca^{2+}; ○; 1E-6M cPV-dimer. The data is the average of 4 determinations. The error bars represent standard error and the solid line represents the line of best fit to the data. Error bars not observed are obscured by the data points.

TABLE 3

KINETIC PARAMETERS OF RAT LIVER GLUTATHIONE REDUCTASE UTILIZATION OF GSSG

	$K_{m,app}$ (M)	$V_{max,app}$ (mol/min/mg)
EDTA	5.01 +/- 0.29E-5	0.02345 +/- 0.0004
Ca^{2+}	6.71 +/- 0.40E-5	0.01014 +/- 0.0003
CaM (1E-6M)	6.80 +/- 0.23E-5	0.01347 +/- 0.0004
ONC-monomer (1E-6M)	6.21 +/- 0.59E-5	0.00716 +/- 0.0004
ONC-monomer (1E-5M)	6.09 +/- 0.55E-5	0.00551 +/- 0.0004
ONC-monomer * (1E-6M)	5.70 +/- 0.24E-5	0.02371 +/- 0.0009
S-carboxymido-ONC (1E-6M)	5.23 +/- 0.17E-5	0.00685 +/- 0.0008
rPV (1E-6M)	7.06 +/- 0.10E-5	0.01029 +/- 0.0012
cPV-dimer (1E-6M)	8.09 +/- 0.08E-5	0.01135 +/- 0.0008

* This run was performed in the presence of 1mM EDTA.

Kinetic parameters obtained are the average of the values obtained for runs of quadruplicate samples. Standard deviation is calculated from the values obtained between runs.

suspected from previous studies (Mutus et al., 1985a). The thiol reacted with DTNB with a second order rate constant of 240+/-5 $M^{-1}min^{-1}$ in Ca^{2+} compared to 24+/- $M^{-1}min^{-1}$ in EGTA. In addition, oncomodulin was found to dimerize in vitro in the presence of Ca^{2+} via its cys-18 thiol. The dimerization reaction was found to be extremely rapid with a k_2 of 1500+/-230 $M^{-1}min^{-1}$. The rate constant of the dimerization reaction was ~6 fold higher than that for the reaction of oncomodulin with DTNB. This suggests that the dimerization reaction is facilitated by the intermolecular attraction of oncomodulin molecules compared to the reaction with DTNB.

ONC-dimer was found to be more calmodulin like in structure than the monomer as evidenced by the relative affinities of the two oncomodulin forms for melittin. Evidence for the interaction of ONC-dimer with melittin was obtained by melittin Sepharose affinity chromatography, melittin tryptophan fluorescence and the use of the Melex competitive binding assay. ONC-dimer was capable of binding to melittin Sepharose in the presence of calcium and could be eluted by the addition of EGTA; ONC-monomer failed to interact with the affinity matrix. Fluorescence studies on the tryptophan emission of melittin indicated that the addition of ONC-dimer to melittin in the presence of calcium resulted in an enhancement and blue shift of the emission maximum, an observation previously reported for the interaction of calmodulin and S100b with melittin.

Competitive binding studies utilizing DNS-CaM illustrated that the dissociation constant for the ONC-dimer melittin complex was 1.1nM compared to 0.9nM for calmodulin. ONC-monomer interacted with melittin with a K_d >1E-6M indicating that the melittin binding domain of ONC-dimer was formed as a consequence of dimerization.

Activity studies with calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, demonstrated that ONC-dimer was more calmodulin like in function than ONC-monomer. ONC-dimer was capable of activating bovine heart phosphodiesterase to ~90% of the calmodulin level of activation with a $K_{activation}$ of 63nM compared to 2nM for calmodulin. ONC-monomer activated PDE but at much higher concentrations with a $K_{activation}$ of 10uM. Only ONC-dimer was capable of activating CaN, with a K_d of 1nM compared to 0.1nM for calmodulin; ONC-monomer had no effect on calcineurin activity. Therefore the elucidation of a melittin binding domain on ONC-dimer in conjunction with the activation of two calmodulin dependent enzymes in a similar concentration range to calmodulin suggests that ONC-dimer is the biologically active species. However, in order for the dimer to be of physiological importance it must be capable of surviving the reducing conditions present *in vivo*. Evidence obtained by SDS-PAGE analysis of the incubation of ONC-dimer with 10mM GSH suggest that the dimer is unstable in the presence of

reducing agents. Therefore, the survival of ONC-dimer *in vivo* remains doubtful.

In contrast to the calmodulin dependent enzymes, the inactivation of glutathione reductase appeared to be a property of ONC-monomer. Both calmodulin and rabbit parvalbumin were unable to alter GSSGRase utilization of GSSG, indicating that the inhibition is an oncomodulin specific event. ONC-monomer was found to be a noncompetitive inhibitor of GSSG utilization indicating that ONC-monomer is binding to a site other than the GSSG binding site. ONC-dimer was found to be a substrate for GSSGRase *in vitro*; this property along with the production of GSH by the enzyme resulting in the conversion of ONC-dimer to monomer made a kinetic analysis of the effect of ONC-dimer on GSSGRase virtually impossible to interpret as the dimer may not be stable through the course of the one minute incubation. The physiological relevance of ONC-dimer as a substrate for GSSGRase is unclear in light of intracellular reducing conditions. However it does indicate that ONC-dimer is capable of interacting with GSSGRase and in this way may alter the activity of the enzyme.

It would appear from these results that both oncomodulin monomer and dimer are capable of interacting with target proteins, some of which may be specific for ONC-monomer or ONC-dimer. ONC-dimer appears to be more structurally related to calmodulin than monomer and as a

result may share some functions with this regulator *in vivo*. In light of the reducing conditions which are maintained intracellularly, the physiological functions of OMC-dimer may exist outside the cell where reducing conditions are less severe. In addition, the results with GSSGRase imply that oncomodulin may have some functions which are not shared by the other members of the Troponin C superfamily, perhaps an indication as to why neoplastic cells express this novel calcium binding regulatory protein.

DISCUSSION

Oncomodulin is a calmodulin like regulatory protein of the beta parvalbumin subclass of the Troponin C superfamily. In addition, oncomodulin has the distinction of being the only Ca^{2+} binding protein that is associated with a diseased state. To date oncomodulin has been found only in neoplastic tissues and more recently in the placenta; these findings indicate that oncomodulin is an oncodevelopmental protein. Therefore, the elucidation of the physiological role of oncomodulin in these systems is of primary importance if one is to unravel the altered metabolism of neoplastic systems.

At the time when this study was undertaken, it was known that oncomodulin was capable of undergoing a Ca^{2+} specific conformational change which is characteristic of other calcium binding regulatory proteins; the closely related parvalbumins could not mimic this conformational flexibility (Mutus et al., 1985a; MacManus et al., 1984; Henzl et al., 1986; Williams et al., 1987). Oncomodulin was also able to stimulate calmodulin dependent enzymes, namely bovine heart and rat heart phosphodiesterase but not bovine brain phosphodiesterase (Mutus et al., 1985b; MacManus, 1981). In addition, oncomodulin was able to stimulate DNA synthesis in Ca^{2+} deprived rat liver cells to a greater

extent than calmodulin (Boynton et al., 1982). Therefore, the role for oncomodulin appeared to be involved with augmenting the effects of calmodulin in neoplastic tissues and hence was part of the mechanism of altered calcium metabolism in tumour cells. However, one thing as yet remained unclear: the concentrations of oncomodulin necessary to activate the calmodulin dependent proteins studied were too high to be of relevance to the in vivo situation (Mutus et al., 1985b; MacManus, 1981).

1. The Dimerization of Oncomodulin

The initial studies undertaken illustrate that oncomodulin is capable of intermolecular dimerization via its cys-18 thiol in vitro. The reactivity of the thiol was determined by studying the rate of reaction with the thiol specific reagent, 5,5'-dithio-(2-nitrobenzoate). The thiol was found to be ~10 fold more reactive toward this reagent in the Ca^{2+} saturated conformation than in the presence of EGTA. These results are in agreement with other work done previously which also illustrates that the thiol is more accessible in the calcium conformation (Mutus et al., 1985a). Studies done on the reactivity of the cys-18 thiol of carp parvalbumin with DTNB indicate that the thiol is more reactive in the absence of calcium (Donato and Martin,

1974). This is a direct indication that although oncomodulin and parvalbumin share extensive homology in primary structure, they are dynamically different proteins.

The kinetics of disulfide bridge formation between oncomodulin molecules was also determined. The second order rate constant for this reaction in the presence of calcium was ~6 fold higher than that observed for the reaction of oncomodulin with DTNB. In the absence of calcium, oncomodulin did not dimerize to any appreciable extent, as only ~5% was dimerized at the end of the 22 hour study. It would appear that, in the calcium conformation, the thiol of oncomodulin is more exposed to the solvent in order for DTNB to react with it. The accessibility of the thiol in the presence of calcium also appears to facilitate the intermolecular dimerization of oncomodulin. The observation that the rate constant of dimerization of oncomodulin was ~6 fold higher than the rate constant for DTNB modification indicates that the dimerization is favoured by oncomodulin in some manner. This is postulated to occur via intermolecular electrostatic interactions between oncomodulin molecules. These attractions would feasibly bring oncomodulin monomers into close proximity with each other thus encouraging dimerization to occur.

2. Interaction of Oncomodulin with Melittin

Oncomodulin, like its parvalbumin relatives, possesses two calcium binding sites compared to calmodulin and troponin C which each have four sites. The full three dimensional structures of calmodulin and troponin C have been determined and illustrate that these proteins are dumbbell shaped, containing two calcium binding domains (each with two calcium binding loops) connected by a long helical segment (Herzberg and James, 1985; Babu et al., 1985 and 1988; Heidorn and Trewhella, 1988). Therefore, one may postulate that upon dimerization, oncomodulin would more closely resemble calmodulin and troponin C in that OMC-dimer would consist of two calcium binding domains, each with two calcium binding loops, joined by a long peptide segment as a result of the intermolecular link.

Basic amphiphilic peptides such as melittin have been utilized as models of the calmodulin binding domain of target proteins (Cox et al., 1985). These peptides have the ability to bind to calmodulin in a calcium specific manner with affinities in the nanomolar range (1-3nM) (Cox et al., 1985; Maulet and Cox, 1983; Comte et al., 1983). These affinities are in the same range as those observed for calmodulin and its target proteins such as phosphodiesterase (1nM) (Kuznicki et al., 1984), calcineurin (<0.1nM) (Hubbard and Klee, 1986), phosphorylase kinase (10nM) (Picton et al.,

1980) and myosin light chain kinase (1.3nM) (Malencik and Anderson, 1982). The interaction of melittin with calmodulin involves the central helix of calmodulin connecting the two dumbbell lobes (Caday and Steiner, 1986). However, there are drawbacks in using basic amphiphilic peptides as models for the calmodulin target protein binding domain (Cox, 1988). Several reports utilizing calmodulin fragments have concluded that the site of interaction on calmodulin is different for different target proteins (Ni and Klee, 1985; Newton et al., 1984; Minowa et al., 1988). In addition, studies in which various amino acid residues of calmodulin have been modified also illustrate differential effects on the interaction with various proteins (Mann and Vanaman, 1988; Giedroc et al., 1985; Guerini et al., 1987). Therefore the use of melittin to study the calmodulin target protein binding domain may be an oversimplification of the various interaction sites of calmodulin for its target proteins. As a result, melittin will be a model for only those target proteins which bind to calmodulin in the same region.

The use of mellitin to detect a calmodulin like binding domain on other proteins such as Troponin C (Steiner and Norris, 1987) and S100 protein (Baudier et al., 1987) is well documented. Therefore, if oncomodulin dimer is more calmodulin like in secondary structure than the monomer, ONC-dimer should display affinities for melittin in the

nanomolar range as does calmodulin.

The interaction of ONC-dimer with melittin was studied in various ways. Firstly, oncomodulin dimer was able to bind to melittin Sepharose in a calcium dependent manner whereas oncomodulin monomer did not interact with the affinity matrix. Secondly, the incubation of ONC-dimer with melittin in a 1:1 molar ratio resulted in an enhancement and blue shift of the tryptophan fluorescence of melittin only when performed in the presence of calcium, an observation which has been previously reported for the interaction of melittin with calmodulin and S100b (Maulet and Cox, 1983; Baudier et al., 1987). The use of melittin Sepharose to study the interaction of calmodulin with melittin was then applied to the search for melittin ONC-dimer interactions. A previous report by Cox and coworkers utilized a competitive binding assay to study the interaction of calmodulin with melittin (Cox et al., 1985). The procedure was modified slightly by using DNS-CaM in the place of radiolabelled calmodulin to compete with the other proteins for melittin. The displacement curves which were obtained in the presence of calmodulin indicated a K_{comp} of 0.6nM, well within the reported range of values (Comte et al., 1983). This indicates that DNS-CaM was able to bind to melittin with the same affinity as native calmodulin. Troponin C was also studied and yielded a K_{comp} of 0.9nM. This result is supported by previous reports of a high

affinity complex between Troponin C and melittin (Steiner and Norris, 1987). ONC-dimer was also able to displace the labelled calmodulin and yielded a K_{comp} of 1.1nM; ONC-monomer was unable to displace sufficient labelled calmodulin for a K_{comp} to be determined. Carp beta parvalbumin was also unable to displace the labelled calmodulin from the melittin Sepharose. Therefore, as a result of the above studies, it is clear that oncomodulin dimer is capable of interacting with melittin with the same affinity as calmodulin whereas oncomodulin monomer lacks this ability. The melittin interaction domain appears to be formed only upon dimerization of oncomodulin since oncomodulin monomer had >100 fold lower affinity for the peptide as estimated from the displacement curves obtained.

These studies indicate that although oncomodulin is parvalbumin like in primary structure, the dimerization of oncomodulin results in a protein which has a high affinity melittin binding site, as calmodulin does. The elucidation of a melittin interaction domain suggests that ONC-dimer may be able to interact with those calmodulin target proteins for which melittin is a model.

3. Interaction of Oncomodulin with Calmodulin Target Enzymes

The elucidation of a melittin binding domain on oncomodulin dimer and not monomer suggests that ONC-dimer has a site of interaction which is similar to that of calmodulin. In order to determine whether this melittin binding domain on oncomodulin dimer results in calmodulin target protein binding properties, the activation of two calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, were studied. Oncomodulin monomer was found to have no effect on calcineurin activity, whereas oncomodulin dimer was found to activate calcineurin to ~80% of the calmodulin level of stimulation with a K_d of 1nM. This was ten fold higher than the reported K_d for calmodulin activation of 0.1nM (Hubbard and Klee, 1986). The activation of phosphodiesterase by oncomodulin dimer occurred with a $K_{activation}$ of 63nM compared to ~10uM for oncomodulin monomer. This observed activation of phosphodiesterase by ONC-monomer is likely due to the dimerization of oncomodulin which would occur during the 30 minute assay. This conclusion is based on the observation that S-acetylated oncomodulin was unable to activate the enzyme. Alternatively, the activation by oncomodulin monomer may be occurring, albeit at lower affinity, and the loss of interaction when oncomodulin was chemically modified

may be a result of denaturation upon chemical modification.

The observed activation of the two enzymes by ONC-dimer indicates that the dimer is interacting with calcineurin with 63 fold higher affinity than with phosphodiesterase. The studies discussed above on the interaction of oncomodulin dimer with melittin suggested that ONC-dimer should interact with calmodulin target proteins in the nanomolar range. However, several reports have been published which suggest that calmodulin has different modes of interaction with its various target proteins (Newton et al., 1984; Klevit and Vanaman, 1984; Putkey et al., 1986; Ni and Klee, 1985; Hanson and Beavo, 1986). On the basis of the study by Ni and Klee, the calmodulin target proteins have been classified into two types (Ni and Klee, 1985). Type I enzymes, which include phosphodiesterase and myosin light chain kinase, bind to multiple domains on calmodulin whereas type II enzymes, such as calcineurin, bind to only one site which is thought to be near the carboxy terminus of calmodulin. This would imply that melittin is a good model for type II enzymes where there is only one site of interaction on the target protein, such as calcineurin. However, it would appear that melittin is not as good a model for type I enzymes where there are multiple sites of calmodulin target protein binding such as phosphodiesterase. Hanson and Beavo (1986) have further subdivided calmodulin dependent phosphodiesterases on the basis of their

calmodulin activation constants. The PDE isozymes studied induced different conformationl changes in calmodulin as evidenced by the affinity of the PDE calmodulin complexes for anti-calmodulin monoclonal antibodies. It is clear from studies such as these that the interaction of calmodulin with target proteins varies with respect to affinity and site(s) of interaction. Therefore, melittin cannot be a model for this complete diversity of interaction domains between calmodulin and its target proteins. In support of this observation, Cachia and coworkers have previously reported that amphiphilic peptides are not good models for calmodulin target protein interactions which involve more than one domain (Cachia et al., 1986).

In view of the above evidence, it would appear that oncomodulin dimer and not monomer is the biologically active species. Only ONC-dimer was capable of interacting with melittin with the same affinity as calmodulin suggesting that oncomodulin dimer has a similar target protein binding domain. This was indeed found to be the case when the activation of phosphodiesterase and calcineurin were studied. ONC-dimer was found to activate both enzymes with affinities in the same range as calmodulin, an ability which oncomodulin monomer lacked. However, in order for oncomodulin dimer to be the physiologically relevant species, the dimerization must be rapid and the dimer must be stable once formed. The dimer indeed does form rapidly

in the presence of calcium with a k_2 of ~1500$M^{-1}min^{-1}$. The cytosol is a highly reducing environment, maintained by millimolar levels of glutathione. These levels are even higher in tumour cells to which oncomodulin is native, as much as 2 to 8 fold (Lee et al., 1987; Russo et al., 1986; Murray et al., 1987). SDS-PAGE experiments on the stability of ONC-dimer to 10mM GSH with respect to time indicate that the dimer is destroyed rapidly under reducing conditions which mimic those of the intracellular environment. Alternatively, it is possible that the reaction between ONC-dimer and GSH was not terminated by lowering the pH and once the sample buffer was added, the denaturing conditions exposed the disulfide to reduction. It would appear, however, that in order for ONC-dimer to exist in vivo, it must be protected in some way from areas of high GSH, possibly an indication that ONC-dimer functions in the extracellular environment, a property that has already been attributed to calmodulin (Crocker et al., 1988). Indeed, both oncomodulin and calmodulin have been shown to stimulate DNA synthesis in rat liver cells when added to the extracellular environment (Boynton et al., 1982). Further evidence of extracellular localization of oncomodulin has been obtained from studies on extraembryonic rat tissues (Brewer and MacManus, 1985). Oncomodulin was detected in the visceral yolk sac which did not have the ability to synthesize the protein, indicating that

oncomodulin had been transported from the site of synthesis and taken up into these cells, a process which is also observed for the oncodevelopmental alpha-fetoprotein (Brewer and MacManus, 1985). It is well documented that some disulfides do exist in the intracellular environment, functioning in the stability of protein structure and also catalysis. For example, glutathione reductase is a disulfide linked dimer <u>in vivo</u> and utilizes thiol disulfide exchange in catalysis (Pai and Schulz, 1983). In addition S100b which is approximately the same size as oncomodulin has been isolated as the disulfide linked dimer (Kligman and Marshak, 1985). Therefore it is possible that ONC-dimer will also be able to withstand the intracellular reducing conditions and remain stable against reduction by GSH.

4. A Potential Regulatory Role for Oncomodulin in Glutathione Metabolism

Reduced glutathione is present in millimolar levels in virtually all cells and is responsible for maintaining intracellular reducing conditions. Glutathione has many functions, one of which is the control of its own biosynthesis through feedback inhibition of gamma-glutamylcysteinyl synthase, the first committed step of the glutathione biosynthetic pathway (Richman and Meister,

1975). In neoplastic tissues, this control mechanism has been altered as several reports have indicated that the total glutathione levels are significantly elevated (Russo et al., 1986; Arrick and Nathan, 1984). These increased levels are thought to afford increased resistivity to chemotherapeutic agents and radiation (Russo et al., 1986; Crook et al., 1986; Arrick and Nathan, 1984) since inhibition of glutathione synthesis resulted in sensitization of the neoplastic cells to antineoplastic treatment (Lee et al., 1987; Russo et al., 1986). An interesting hypothesis arose out of these findings: if one could selectively alter tumour glutathione levels relative to normal cells, antineoplastic treatment would feasibly kill neoplastic tissue while leaving normal cells virtually unharmed.

Glutathione reductase is central for maintaining the high ratio of reduced to oxidized glutathione intracellularly. This enzyme is responsible for the conversion of oxidized glutathione formed upon the action of glutathione peroxidase which converts hydrogen peroxide to water thus decreasing the effects of several toxic agents. In addition, glutathione reductase has been illustrated to be an inducible enzyme when rat liver cells were treated with various compounds (Carlberg et al., 1981). This suggests that glutathione reductase is of importance to the protection of cells against toxic agents.

The possibility of the involvement of oncomodulin in the regulation of GSSGRase was first suspected on the basis of SDS-PAGE studies. SDS-PAGE experiments of oxidized oncomodulin and glutathione reductase illustrated that ONC-dimer can act as a substrate for this enzyme in vitro. Overnight incubation of ONC-dimer with GSSGRase in the presence and absence of NADPH resulted in the essentially quantitative conversion of oncomodulin dimer to monomer. Incubation of ONC-dimer either alone or with NADPH resulted in no conversion to monomer. Therefore GSSGRase was essential to affect to conversion of ONC-dimer to monomer and was using ONC-dimer as a substrate. In light of the known mechanism for glutathione reductase activity, NADPH is expected to be essential to the reduction (Pai and Schulz, 1983). The contamination of endogenous NADPH in the enzyme preparation cannot be ruled out since the enzyme was used as obtained. Indeed, the published purification procedure for GSSGRase employs the elution of the enzyme from 2',5'-ADP Sepharose with an NADPH gradient (Carlberg and Mannervik, 1985). Fluorescence measurements of the GSSGRase used illustrated fluorescence emission at 460nm when excited at 340nm; this is characteristic of NADPH fluorescence. The amount of NADPH contaminating the purchased enzyme preparation and also our purified preparation from rat liver was in excess on a per mole basis to the amount of enzyme present. Dialysis and Sephadex G-25 chromatography were

unable to remove the NADPH from the enzyme suggesting a strong interaction between the two. Therefore the amount of NADPH present in the enzyme preparation was sufficient to allow for the reduction of ONC-dimer in the absence of added NADPH. Subsequent studies on the time dependence of the reduction of ONC-dimer by equimolar amounts of GSSGRase indicate that the reaction is relatively rapid being complete within ~5 minutes. The physiological relevance of ONC-dimer acting as a substrate for GSSGRase is unclear in view of intracellular reducing conditions. It is however an indication that ONC-dimer is capable of interacting with GSSGRase and as a result may have an effect on the enzyme's activity.

The interaction of oncomodulin with GSSGRase was also studied utilizing oncomodulin-Sepharose. Glutathione reductase (from bovine intestinal mucosa) was found to bind to the affinity column in the presence of Ca^{2+}. The enzyme could then be eluted, fully active, from the column by the application of EGTA. Therefore the interaction of glutathione reductase with reduced oncomodulin is a Ca^{2+} dependent process. Glutathione reductase was unable to bind to calmodulin Sepharose under identical conditions. Additional evidence for the interaction of ONC with GSSGRase was observed when glutathione reductase (from bovine intestinal mucosa and also rat liver) was assayed in the presence of increasing amounts of reduced oncomodulin. ONC-

monomer was able to inhibit glutathione reductase with 50% inactivation occurring for the rat liver enzyme at ~1.3E-5M and for the bovine enzyme at ~1E-5M. Again the interaction was calcium dependent as no inactivation occurred in the presence of oncomodulin monomer in EDTA. The level of oncomodulin in rat Morris hepatomas is ~100 mg/kg or 1E-5M suggesting that this inhibition is physiologically possible (MacManus, 1981b). Calmodulin and rabbit parvalbumin were unable to alter glutathione reductase activity under identical conditions. This is the first report of a regulatory mechanism for oncomodulin which is not also a property of calmodulin. Since oncomodulin is oncodevelopmental in origin, this would imply that this inhibition would occur upon neoplastic transformation. This feasibly could result in a different equilibrium point between oxidized and reduced glutathione compared to normal cells.

In order to determine the mode of interaction of oncomodulin with GSSGRase a kinetic study was undertaken. The kinetics of oxidized glutathione utilization in the presence of various Ca^{2+} binding proteins was determined. Calcium itself resulted in a decrease in the apparent V_{max} in comparison to EDTA but no change in the apparent K_m for either the rat liver or bovine intestinal mucosa enzymes. This result was to be expected as the published purification procedures and assays employ the use of EDTA in all buffers

(Carlberg and Mannervik, 1985; Carlberg and Mannervik, 1981; Carlberg et al., 1981).

Calmodulin is known to be involved in the regulation of several cellular processes. Its effect on glutathione reductase activity from both sources, however, was identical to that seen in Ca^{2+} alone and therefore had no effect on GSSG utilization. This was expected from the titration curves obtained in the presence of CaM and also the results of calmodulin Sepharose chromatography.

In contrast to calmodulin, reduced oncomodulin interacted with glutathione reductase significantly lowering the apparent V_{max} for GSSG utilization while the apparent K_m was unaffected for both enzyme preparations. Therefore, reduced oncomodulin is acting as a noncompetitive inhibitor of GSSG utilization by glutathione reductase. This implies that ONC-monomer is binding to the reductase at a site other than the active site resulting in a conformational change in GSSGRase thus decreasing its activity. In tumourous tissues, this inhibition would translate into a decrease in the ratio of reduced to oxidized glutathione thus resulting in lower reducing capability. Reduced glutathione would feasibly then increase as a result of the removal of the negative feedback inhibition of gamma-glutamylcysteinyl synthase, hence increasing total glutathione levels. In order to determine whether the cys-18 thiol of oncomodulin was involved in the inactivation, the effect of SAMONC on

GSSG utilization was studied. SAMONC was capable of inhibiting GSSGRase from both sources to the same extent as reduced oncomodulin suggesting that the free thiol is not necessary for the inhibition. Previous work has suggested that the free thiol of oncomodulin was necessary for the activation of bovine heart phosphodiesterase as blocking of the thiol with iodoacetamide abolished the activation (Mutus et al., 1988). The affinity of ONC-monomer for GSSGRase is approximately the same as that obtained for bovine heart phosphodiesterase since half maximal activation of PDE occurred at ~1E-5M as does 50% inhibition of GSSGRase (Mutus et al., 1985b). Therefore, the site of interaction of bovine heart phosphodiesterase with oncomodulin is different or at least more sensitive to structural perturbations than that to which GSSGRase binds.

The possibility that the observed inhibition was due to oncomodulin sequestering NADPH was also considered. Studies on the effect of ONC-monomer on NADPH fluorescence illustrated a 5% increase in the fluorescence intensity with no apparent change in the emission wavelength maximum. A similar effect was observed in the presence of equimolar amounts of rabbit parvalbumin which is not capable of GSSGRase inhibition. Further evidence that ONC-monomer is not sequestering NADPH is illustrated in FIGURE 46. The reaction of GSSGRase is a compulsory order two substrate mechanism. Theoretical plots at various fixed

concentrations of NADPH illustrate that the Lineweaver-Burke plots obtained have an altered K_m and V_{max}. In the presence of ONC-monomer, only the apparent V_{max} has been altered, thus illustrating that the inhibition of GSSGRase by ONC-monomer is not a result of the removal of NADPH.

From the results of SDS-PAGE previously discussed, it was concluded that ONC-dimer is a substrate for GSSGRase in vitro. In addition, during the course of the kinetic assay, GSH will be formed by GSSGRase which feasibly can interact with ONC-dimer, causing its reduction. Therefore it is not clear how long ONC-dimer would survive during the assay. It is apparent that once ONC-monomer is formed, it is capable of inhibiting the enzyme. The formation of ONC-monomer will also result in the concomitant formation of GSSG which can be utilized again by GSSGRase. Additive to this is the binding of ONC-dimer to the active site of the enzyme. As a result of these various factors, the effect of ONC-dimer on GSSGRase activity could not be studied by this method. The intracellular concentration of ONC-dimer is expected to be much lower than that used for the ONC-monomer kinetics (1E-6M) as a result of the highly reducing conditions present intracellularly. Therefore the physiological effect of ONC-dimer on glutathione reductase activity will be minimal compared to that of ONC-monomer.

Carp beta parvalbumin is also capable of dimerization via its cys-18 thiol. The kinetics obtained in the presence

of cPV-dimer were identical to those observed in the presence of Ca^{2+} alone and therefore had no effect on GSSGRase activity from either enzyme source. As discussed for ONC-dimer, it is unclear whether the observed kinetics are a result of cPV-dimer or monomer. Rabbit beta parvalbumin is ~50% homologous to rat oncomodulin but lacks the cys-18 thiol. Rabbit parvalbumin also had no effect on GSSGRase utilization of GSSG. Therefore the site of interaction with GSSGRase on oncomodulin is lacking in the carp and rabbit parvalbumins. Since chemical modification of the cys-18 thiol of oncomodulin had no effect on inhibitory ability, the fact that rabbit parvalbumin has a phenylalanine at this position should be irrelevant. It is possible that the site of interaction on oncomodulin may be exposed only upon the conformational change which occurs upon the binding of Ca^{2+} since the inhibition was not observed in EDTA; the parvalbumins do not undergo a Ca^{2+} specific conformational change and thus the site of interaction may be inaccessible (Mutus et al., 1985a).

Therefore, the inhibition of GSSGRase appears to be an oncomodulin specific process as neither calmodulin nor parvalbumin (carp or rabbit) were able to alter GSSGRase activity. The interaction of oncomodulin with GSSGRase was observed by SDS-PAGE, oncomodulin-Sepharose chromatography and enzyme activity studies. The observed kinetics in the presence of ONC-monomer were characteristic of

noncompetitive inhibition. The effect of ONC-dimer on GSSGRase activity could not be accurately assessed as a result of the instability of ONC-dimer to GSH formed during the assay and also the conversion of ONC-dimer to monomer by the enzyme itself.

CONCLUSIONS

Evidence has been provided for the *in vitro* dimerization of oncomodulin. The disulfide linked dimer more closely resembles calmodulin as illustrated by its melittin binding affinity. The finding of a melittin binding domain on oncomodulin dimer and not monomer implies that ONC-dimer possesses a calmodulin like target protein binding domain. In support of this, ONC-dimer was shown to activate two calmodulin dependent enzymes, bovine heart phosphodiesterase and bovine brain calcineurin, in a similar concentration range as that observed for calmodulin. ONC-monomer was unable to activate calcineurin in the range tested and only activated phosphodiesterase at much higher concentrations. These results led to the conclusion that oncomodulin dimer is the biologically active species (Mutus et al., 1988). Studies on the kinetics of dimerization indicated that the reaction was rapid in the presence of calcium. However, oncomodulin dimer is not expected to exist for extended periods of time intracellularly due to the reducing conditions which exist. A time study on the stability of ONC-dimer to 10mM GSH indicated that the dimer was unable to survive the reducing conditions. In view of these facts, the search for oncomodulin dimer dependent processes may be more profitable in the extracellular environment where these strong reducing conditions do not

exist. To this end, calmodulin and oncomodulin have previously been shown to stimulate DNA synthesis when added to the extracellular environment in vitro (Boynton et al., 1982; Crocker et al., 1988). In addition, oncomodulin has been illustrated to be transported from its site of synthesis in the placenta (Brewer and MacManus, 1985) further suggesting that an extracellular role for oncomodulin in vivo is possible.

Evidence has also been provided for the enzymatic conversion of oncomodulin dimer to monomer by glutathione reductase in vitro. The physiological significance is again unclear in view of intracellular reducing conditions. In addition, oncomodulin monomer was found to inhibit GSSGRase. The concentrations of monomer used for the studies are well within the range of the concentration of oncomodulin monomer reported in vivo (MacManus, 1981). This inhibition was determined to be oncomodulin specific as neither calmodulin or parvalbumin (from rabbit or carp) were able to alter GSSGRase activity. This is the first report of a regulatory action of oncomodulin which is not a function of calmodulin.

It is probable that oncomodulin has many regulatory roles in tumour cells which are as yet unknown. These processes may require the action of oncomodulin monomer and/or dimer. It is suggested from the above results that oncomodulin dimer is more closely related to calmodulin than monomer and hence it was concluded that the dimer was the

biologically active species. However, no research has been done to date on target proteins isolated from tumour tissue. The calcium regulation of neoplasms is different from that of normal cells. Therefore it is possible that being calmodulin like is not advantageous to all target enzymes and reduced oncomodulin may be of more significance to some processes. Indeed, in the case of glutathione reductase, an enzyme which is thought to be central to the altered metabolism of glutathione in neoplasms, being calmodulin like is clearly not an advantage since calmodulin had no effect on GSSG utilization by the enzyme.

In view of these findings, it appears likely that both oncomodulin monomer and dimer have regulatory roles in tumour cells, some of which are different from each other. The results suggest that oncomodulin monomer is involved in the regulation of glutathione levels in tumour cells via the inhibition of GSSGRase, an enzyme which is important in the survival of tumour cells upon antineoplastic treatment. The expression of oncomodulin upon neoplastic transformation indicates that oncomodulin is advantageous to these systems, which already possess calmodulin. Therefore it is not surprising and indeed is to be expected that oncomodulin will have functions which are not shared by calmodulin. The elucidation of the roles of oncomodulin in tumours is therefore essential if one is to understand the altered metabolism of neoplastic systems and ultimately be able to

control their proliferation.

APPENDIX 1

EGTA Buffering

In order to attain calcium concentrations in the 1E-10 to 1E-6M range, EGTA buffering was used. The method employed is that of Sillen and Martell (1971). Chelex water was utilized for all of the following.

The dissociation constant for the binding of Ca^{2+} to EGTA is defined as follows:

(1) $$K_d = [Ca^{2+}][EGTA]/[Ca^{2+} \cdot EGTA]$$

where, $[EGTA_T] = [EGTA] + [Ca^{2+} \cdot EGTA]$

and $[Ca^{2+}_T] = [Ca^{2+}]_{free} + [Ca^{2+} \cdot EGTA]$

It is assumed that a) $[Ca^{2+}]_{free} <<< [Ca^{2+} \cdot EGTA]$.

b) $[EGTA] = [EGTA]_T - [Ca^{2+} \cdot EGTA]$

Therefore,

(2) $$[Ca^{2+}]_{free} = K_d[Ca^{2+} \cdot EGTA]/[EGTA]$$

The following equation is used to calculate the association constant for EGTA and Ca^{2+}:

(3) $$K/K' = 1 + K_1[H^+] + K_1K_2[H^+]^2 + K_1K_2K_3[H^+]^3 + K_1K_2K_3K_4[H^+]^4$$

where K_1, K_2, K_3, K_4 are the K_m's for the various sites on EGTA for calcium binding ($pK_1 = 9.46$; $pK_2 = 8.85$; $pK_3 = 2.65$; $pK_4 = 2.0$). The last two terms of equation 3 are considered negligible and are therefore omitted. K, the association constant, for a solution of 0.1 ionic strength was given as 9.33E10. Using these values, a value for K' can be calculated. Therefore, one can calculate K_d for the Ca^{2+} EGTA complex according to the following equation:

(4) $$K' = 1/K_d$$

The value of K' can also be corrected for ionic strength according to the following equation:

(5) $$\log K'_{I=0.15} = \log K'_{I=0.1}(0.15/0.1)$$

Therefore, one can calculate K' at any ionic strength and utilizing equation 4, calculate the corresponding K_d.

Using equation 2, one may then calculate $[Ca^{2+}]_{free}$, assuming that $[EGTA]_T - [Ca^{2+} \cdot EGTA] = [EGTA]$ and $[Ca^{2+}]_T = [Ca^{2+} \cdot EGTA]$.

Therefore,

(6) $$[Ca^{2+}]_{free} = K_d[Ca^{2+}]_T/([EGTA]_T - [Ca^{2+}]_T)$$

Utilizing this method, free Ca^{2+} concentrations of 1E-10 were attainable.

APPENDIX 2

DETERMINATION OF FRACTION BOUND IN THE MELEX COMPETITIVE BINDING ASSAY

In the competitive binding assay used for the study of the interaction of calcium binding proteins with Melex, the following equilibria are obtained:

$$(1) \qquad M + CaM \underset{}{\overset{K_c}{\rightleftharpoons}} M \cdot CaM$$

$$(2) \qquad M + Comp \underset{}{\overset{K_{comp}}{\rightleftharpoons}} M \cdot Comp$$

where Comp refers to the competing protein, M refers to Melex and K_c and K_{comp} are the dissociation constants for the corresponding reactions.

The fraction of DNS-CaM bound is defined as:

$$(3) \qquad F_b = [M \cdot DNS\text{-}CaM] / [M]_T$$

where,

$$(4) \qquad [M]_T = [M \cdot DNS\text{-}CaM] + [M]_{free} + [M \cdot Comp]$$

Utilizing the equations for the two equilibria:

$$(5) \qquad K_c = [M][DNS\text{-}CaM] / [M \cdot DNS\text{-}CaM]$$

(6) $$K_{comp} = [M][Comp]/[M \cdot Comp]$$

and substituting into equation 3, the following equation for the determination of the fraction bound is obtained:

(7) $$F_b = [DNS\text{-}CaM]/(K_c([DNS\text{-}CaM]/K_c + 1 + [Comp]/K_{comp}))$$

Therefore by utilizing the value obtained by Cox and coworkers for K_c of 1nM (Cox et al., 1985), the value for K_{comp} for various proteins can be obtained by a fit of equation 7 to the DNS-CaM displacement curve obtained.

APPENDIX 3

THEORETICAL EFFECT OF ONCOMODULIN-MONOMER SEQUESTERING OF NADPH

The catalytic mechanism of GSSGRase is a compulsory order two substrate reaction. The Michaelis Menton equation which describes the kinetics of this system is that of a Ping Pong Bi Bi system:

$$(1) \qquad v = \frac{V_{max}[GSSG][NADPH]}{K_{m,G}[NADPH] + K_{m,N}[GSSG] + [GSSG][NADPH]}$$

This equation has been demonstrated to fit the kinetics obtained with GSSGRase when the substrate ranges are bounded by [NADPH]: 2 - 60uM and [GSSG]: 2 - 100uM (Carlberg and Mannervik, 1975). The reported values for the kinetic parameters for the rat liver enzyme are: $K_{m,a}$ = 8.2uM; $K_{m,b}$ = 26.3uM. The equation for the corresponding Lineweaver Burke plot is as follows:

$$(2) \qquad 1/v = \frac{K_{m,G}}{V_{max}} \cdot \frac{1}{[GSSG]} + \frac{K_{m,N} + [NADPH]}{V_{max} * [NADPH]}$$

Utilizing a maximal velocity where both substrates are saturating of 100umol/min/mg three values of [NADPH] were chosen to simulate the effect of oncomodulin monomer removing NADPH from the reaction (1uM, 2uM and 5uM). These values were then substituted into equation 2. The theoretical lines (FIGURE 46) were then obtained by substituting in values for [GSSG] and determining the value of v. The Lineweaver Burke plots which are obtained by this method are parallel to each other indicating a change in both $K_{m,app}$ and $V_{max,app}$.

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